Departamento de Farmacología
Facultad de Farmacia
Universidad de Sevilla

CARACTERIZACIÓN DE LAS PROPIEDADES FUNCIONALES A NIVEL CARDIOVASCULAR Y DEL SÍNDROME METABÓLICO DE UN EXTRACTO ENZIMÁTICO DE SALVADO DE ARROZ

Tesis Doctoral presentada por

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Directoras

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Sevilla, 2014

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Memoria presentada por **Mª Luisa Justo Gómez** para optar al grado de Doctora en Farmacia, con Mención Internacional.

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Sevilla, 2014

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Dña. Mª DOLORES GARCÍA GIMÉNEZ, Profesora Catedrática de Farmacología y Directora del Departamento de Farmacología de la Facultad de Farmacia de la Universidad de Sevilla,

CERTIFICA:

Que la presente Tesis Doctoral titulada "CARACTERIZACIÓN DE LAS PROPIEDADES FUNCIONALES A NIVEL CARDIOVASCULAR Y DEL SÍNDROME METABÓLICO DE UN EXTRACTO ENZIMÁTICO DE SALVADO DE ARROZ" realizada por **Mª LUISA JUSTO GÓMEZ** ha sido dirigida por la **Dra. Mª Dolores Herrera González** y la **Dra. Rosalía Rodríguez Rodríguez** para aspirar al grado de Doctora en Farmacia con Mención Internacional, cumpliendo los requisitos para este tipo de trabajo.

Y para que así conste, firmo la presente.

En Sevilla, a 16 de Julio de 2014

Fdo.: Dña. Mª Dolores García Giménez

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CERTIFICAN,

Que la presente Tesis Doctoral titulada "CARACTERIZACIÓN DE LAS PROPIEDADES FUNCIONALES A NIVEL CARDIOVASCULAR Y DEL SÍNDROME METABÓLICO DE UN EXTRACTO ENZIMÁTICO DE SALVADO DE ARROZ" realizada por **Mª LUISA JUSTO GÓMEZ**, para aspirar al grado de Doctora en Farmacia con Mención Internacional, ha sido llevada a cabo bajo su dirección.

En Sevilla, a 16 de Julio de 2014

Vº Bº de los Directores

Fdo.: Dra. Mª Dolores Herrera González

Fdo.: Dra. Rosalía Rodríguez Rodríguez

El doctorando

Fdo.: Mª Luisa Justo Gómez

Todo aquél que me conoce sabe que soy más de demostrar afecto que de escribirlo, así que comenzaré pidiendo perdón. Perdón a todas aquellas personas que esperaban poder buscar su nombre y ver unas palabras dedicadas, porque no las encontrará en este caso.

Pienso que cuando se presta servicio a alguien o a un colectivo, el mejor reconocimiento que se puede tener es poder ver los beneficios que se le ha aportado al/los destinatarios de la acción o servicio prestado. Por tanto, en esta ocasión, quiero mostrar mi profundo agradecimiento a quienes me han ayudado en este camino enumerando algunos de los aportes que han hecho a mi persona, y a su vez, a la elaboración de este trabajo científico. No habrá nombres, sino acciones con las que cada uno podrá sentirse identificado:

- A quien tuvo la intuición de escogerme a mí entre varias opciones, porque me ha permitido vivir esta aventura y una etapa que, sin duda, marcará mi vida. Gracias.

- A quien ha logrado sacar lo mejor de mí, y se ha preocupado de que viva la tesis como un camino de autoconocimiento y superación, lucha y disfrute, porque este aprendizaje me lo llevo para toda la vida. Gracias.

- A quien ha sabido estar atento/a para ofrecer su ayuda, aunque fuera en lo más mínimo, porque todo pequeño detalle forma parte después de algo más grande. Gracias.

- A quien ha sabido ser compañero/a, amigo/a, terapeuta, payaso/a…lo que hiciera falta cuando hiciera falta, porque han visto mis defectos y limitaciones, y los han acogido y cuidado para hacerme fuerte frente a la adversidad. Gracias.

- A quien se ha mantenido cerca de mis alegrías, retos y triunfos en lo cotidiano, porque no sólo me han hecho sentirme especial, sino que me han enseñado a hacer que el otro también se sienta especial cada día. Gracias.

- A quienes me han aguantado en la intimidad del hogar, porque también han prestado el servicio más difícil: el amor incondicional. Gracias.

"No importa cuánto se viva, sino cómo se viva. Si se vive bien y se muere joven, se puede haber contribuido más que una persona hasta los ochenta años preocupada sólo de sí misma."

Martin Luther King

A mis padres,

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INTRODUCCIÓN

1. EL SÍNDROME METABÓLICO COMO FACTOR DE RIESGO CARDIOVASCULAR.

Cuando hablamos de las **enfermedades cardiovasculares** nos referimos a un conjunto de trastornos del corazón y de los vasos sanguíneos, entre los que se incluyen patologías como la hipertensión arterial, la cardiopatía coronaria o infarto de miocardio, los accidentes cerebrovasculares, la enfermedad vascular periférica, la insuficiencia cardíaca, la cardiopatía reumática y congénita y las miocardiopatías. Las enfermedades cardiovasculares son la principal causa de defunción en todo el mundo. Según la Organización Mundial de la Salud (OMS), se calcula que en 2012 han fallecido más de 17 millones de personas por este tipo de patologías, lo cual representa el 31 % de las defunciones registradas en el mundo. De esas defunciones, aproximadamente 7,3 millones se debieron a cardiopatías coronarias, y 6,7 millones a accidentes cerebrovasculares. Hoy día estas patologías se distribuyen en todo el mundo, afectando casi por igual a hombres y mujeres, y se prevé que sigan siendo la principal causa de muerte en los próximos años. [1]

Una de las condiciones que se encuentran más ligadas a las enfermedades cardiovasculares es la **obesidad**. Según los datos de la OMS, la obesidad y el sobrepeso han alcanzado caracteres de epidemia a nivel mundial. Más de mil millones de personas adultas tienen sobrepeso y, de ellas, al menos 300 millones son obesas. Hay lugares como EEUU donde, según las estadísticas del año 2012, más de 35 % de la población adulta y casi el 20 % de la juventud sufren obesidad. En el caso de España, la prevalencia de obesidad en la población adulta (25-60 años) es del 14,5 % mientras que el sobrepeso asciende al 38,5 %. La obesidad es más frecuente en mujeres (17,5 %) que en varones (13,2 %). También se ha observado que la prevalencia de obesidad crece conforme aumenta la edad de las personas, alcanzando cifras cercanas al 30 % a partir de 55 años, lo que afecta cada vez más a la esperanza de vida del país. Más preocupante resulta este fenómeno en la población

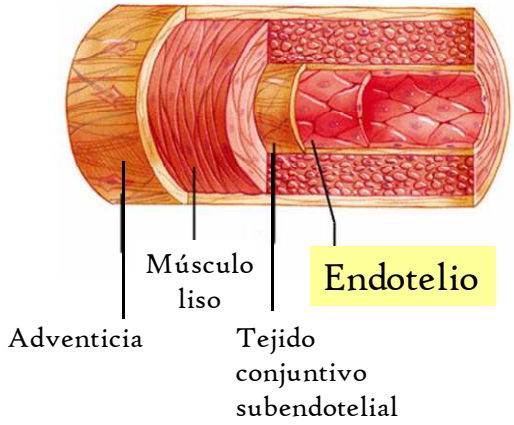
infantil y juvenil, ya que se calcula que el 8,5 % de los españoles de entre 2 a 17 años presenta obesidad y el 18,2 % tiene sobrepeso. Las mayores cifras de obesidad se detectan en la prepubertad y, en concreto, en el grupo de edad de 6 a 12 años, con una prevalencia del 16,1 %. En comparación con el resto de Europa, España se sitúa en una posición intermedia en el porcentaje de adultos obesos. Sin embargo, en lo que se refiere a la población infantil, nuestro país presenta una de las cifras más altas, sólo comparable a las de otros países mediterráneos como Italia, Malta y Grecia [2,3]. Además, como veremos a continuación, el incremento de la obesidad como epidemia ha provocado un incremento en las complicaciones que históricamente se han visto asociadas al desarrollo de esta patología. Aparte del descomunal coste económico que implica en los sistemas nacionales de salud y el estigma social que causa, la obesidad aumenta el riesgo de muerte prematura y de desarrollar otras enfermedades como diabetes mellitus tipo 2 (que por su alta asociación ya se habla del término "*Diabesity*"), enfermedad cardiovascular, apnea del sueño obstructiva, enfermedad del hígado graso no-alcohólica así como diversos tipos de cáncer. Se calcula que la prevalencia de obesidad en el mundo continuará creciendo de forma exponencial y superará los 1.500 millones de personas en 2015. Para ser más concretos, podemos decir que la obesidad constituye la segunda causa de muerte previsible después del tabaco, pues cada año fallecen 2,8 millones de adultos en todo el mundo. Según los estudios, la obesidad presenta el 44 % de la morbilidad asociada a diabetes, el 23 % a cardiopatías isquémicas y entre el 7 % y el 41 % de la que se encuentra asociada a determinados cánceres. [1,4]

Dentro de las enfermedades cardiovasculares, la obesidad establece estrechos lazos de unión con otras patologías que también constituyen importantes factores de riesgo cardiovascular. Un ejemplo claro de esto es la relación que existe entre el individuo obeso y su probabilidad de desarrollar **diabetes mellitus tipo 2**. La obesidad se considera el principal factor de riesgo para padecer esta enfermedad, ya que el 70-90 % de los pacientes diabéticos no insulino-dependientes son obesos. Por

cada kilogramo de peso ganado, el riesgo de desarrollar diabetes aumenta un 9 % [5]. La prevalencia de **diabetes tipo 2** ha aumentado rápidamente en todo el mundo y se estima que en 2030 más de 350 millones de personas la padecerán [6]. Además del coste sanitario que esta patología supone para la población, estos pacientes ven incrementado a su vez el riesgo de padecer enfermedades cardiovasculares, algunos tipos de cáncer, y enfermedades a nivel ocular y renal [7]. La **insulina**, como hormona producida por las células β-pancreáticas, desempeña un papel fundamental en el metabolismo de los hidratos de carbono y de las grasas. Esta hormona regula el consumo de la glucosa plasmática por parte de los tejidos, entre los que se incluye el hígado y el músculo esquelético. En el individuo obeso este equilibrio metabólico se ve comprometido, ya que se producen alteraciones tanto en la absorción de la glucosa como en la sensibilidad a la insulina por parte de los tejidos [8]. Otro de los factores patogénicos más estudiados y que en un principio puede llevar a insulinorresistencia y a la disfunción de las células β-pancreáticas, disminuyendo la tolerancia a la glucosa y, en última instancia, produciendo diabetes tipo 2, es el **estrés oxidativo**. Este mismo mecanismo se encuentra implicado en las alteraciones macro y microvasculares asociadas a la diabetes tipo 2, y es por ello que los sujetos con diabetes tipo 2 ven mermada su capacidad de superar el colapso generado por el incremento de radicales libres (*reactive oxygen species*, ROS) [9]. A su vez, la hiperinsulinemia presente en la obesidad (y en su evolución y agravamiento por su coexistencia con otras patologías que, como se comentará más adelante, se ha venido a llamar *síndrome metabólico*) se relaciona con una pérdida de elasticidad por parte de las arterias, sobre todo porque alteraciones como la intolerancia a la glucosa provoca un deterioro en la función arterial que hace a estos pacientes más propensos a desarrollar **hipertensión arterial**. Además, la retención de sodio por parte del organismo obeso puede agravar esta situación con una hiperreactividad del sistema nervioso simpático, en la que están implicados

mecanismos ligados a la síntesis de angiotensinógeno, angiotensina II y la función endocrina del tejido adiposo. [10,11].

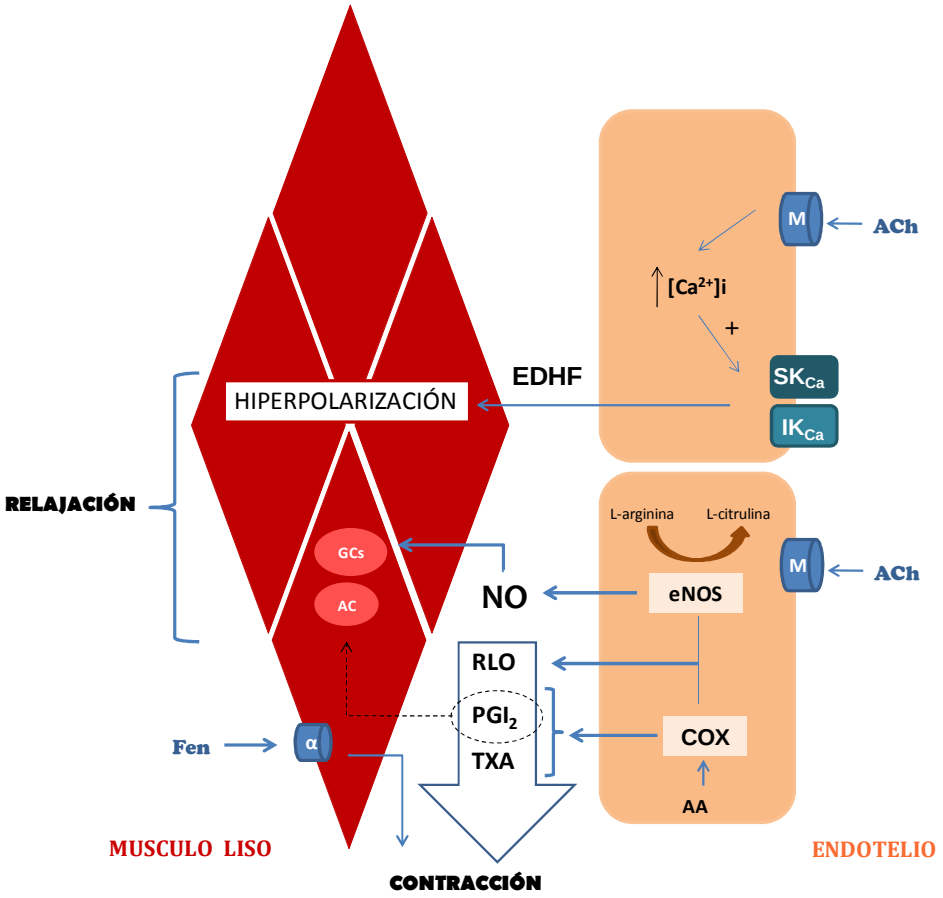
Fig. 1 Fisiología del vaso arterial.



Por tanto, el individuo obeso con frecuencia va a desarrollar alteraciones de tipo vascular, en la que varios mecanismos se encuentran involucrados, jugando un papel fundamental el intercambio de sustancias a través de la barrera endotelial. Cuando hablamos de la fisiología de los vasos sanguíneos, adquiere especial relevancia una de las barreras fisiológicas del organismo más involucradas en la patología cardiovascular asociada a la obesidad: **el endotelio vascular**. Debemos tener en cuenta que una arteria está formada por tres capas principalmente: la capa interna o túnica íntima, formada por una mono-capa de células endoteliales que están en contacto directo con la sangre; la capa o túnica media, constituida por células de músculo liso integradas en la matriz extracelular; y la adventicia, la capa más externa, que contiene mastocitos, terminaciones nerviosas y microvasos (Fig. 1) [12]. El endotelio vascular juega un papel fundamental en la regulación del tono vascular por ser responsable de la liberación de multitud de sustancias vasoactivas que favorecen la distribución del flujo sanguíneo según la demanda de los distintos tejidos. Una de las más destacables es el óxido nítrico (*nitric oxide*, NO), producido en condiciones fisiológicas por la enzima óxido nítrico sintasa endotelial (*endotelial*

nitric oxide synthase, eNOS), el cual, junto a la resistencia vascular y a la activación de receptores, es el máximo responsable de la homeostasis vascular. El NO produce la relajación de las células musculares lisas mediante la guanilil-ciclasa (GC) y el incremento de la producción de guanosín monofosfato cíclico (GMPc) (Fig. 2) [13]. Sin embargo, la importancia del NO no sólo se debe a su actividad vasodilatadora, sino a su acción inhibitoria frente a mecanismos vasoconstrictores, agregación plaquetaria y proliferación de células del músculo liso vascular. Debido a la naturaleza multifuncional de esta molécula, una disminución de su biodisponibilidad, junto con un incremento de esta misma molécula producida en exceso mediante la isoforma inducible (iNOS) y otros mediadores producidos por el endotelio responsables de la vasocontricción, como es la endotelina-1, especialmente presente en los procesos de hipertensión, fallo cardíaco o aterosclerosis, se consideran factores de riesgo para el desarrollo de enfermedades cardiovasculares de mayor gravedad (Fig. 2) [14]. Otros de los mecanismos reguladores de la función vascular vienen determinados por el nivel en sangre de diferentes moléculas como puede ser la adiponectina. Se sabe que el nivel de adiponectina sérica está muy relacionado con la capacidad vasorrelajante dependiente de endotelio, ya sea en la respuesta mediada por la liberación de NO, o aquella mediada por los canales de potasio dependientes de calcio (*calcium-dependent potassium channels*, K_{Ca}), considerados responsables de la liberación del factor hiperpolarizante dependiente de endotelio (*endothelium-dependent hyperpolarizing factor*, EDHF). La adiponectina también se encuentra involucrada en procesos liderados por factores independientes de endotelio, como los derivados de la ciclooxigenasa-2 o los mediadores que son activados por citoquinas como el factor de necrosis tumoral-alfa (*tumor necrosis factor-alpha*, TNF-α) (Fig. 2) [15, 16].

Fig. 2 Esquema de los procesos moleculares que tienen lugar en el endotelio y en el músculo liso vascular.



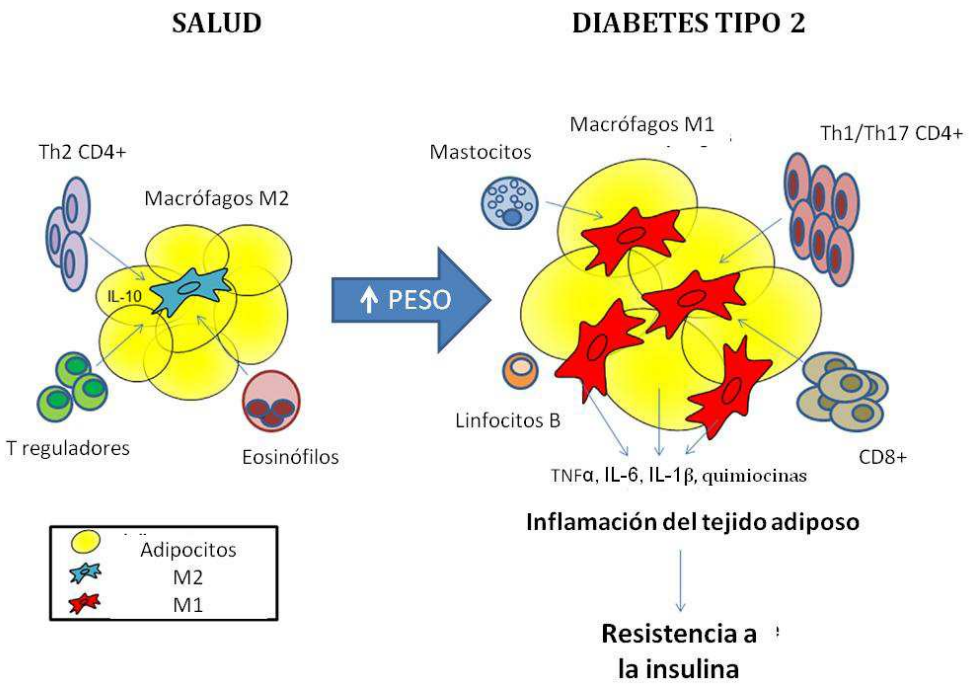
α: receptor alfa-adrenérgico; **AA**: ácido araquidónico; **AC**: adenil ciclasa; **ACh**: acetilcolina; **[Ca^{2+}]i**: concentración de calcio intracelular; **COX**: cicloooxigenasa; **EDHF**: Factor hiperpolarizante dependiente de endotelio; **eNOS**: óxido nítrico sintasa endotelial; **Fen**: fenilefrina; **GCs**: guanilil ciclasa; **IK_{Ca}, SK_{Ca}**: canales de potasio dependientes de calcio de intermedia y baja conductancia; **M**: receptor muscarínico; **NO**: óxido nítrico; **PGI_2**: prostaglandina I_2; **RLO**: radicales libres de oxígeno; **TXA**: tromboxano A.

Si seguimos en la línea de averiguar qué procesos se desencadenan en el individuo sano que ha alcanzado un grado de sobrepeso considerable, aumentando con ello la grasa acumulada, también podemos hablar de la grasa que rodea a los vasos sanguíneos. La mayoría de los vasos sanguíneos están rodeados por una variable cantidad de tejido adiposo adventicio, llamado tejido adiposo perivascular (*perivascular adipose tissue*, **PVAT**), el cual se consideraba hasta hace poco más de una década un mero soporte mecánico para el vaso arterial. Ahora se sabe que, al igual que la grasa que se encuentra en el resto del organismo, este tejido también es dinámico y secreta sustancias bioactivas como el factor de crecimiento del endotelio vascular (*vascular endotelial growth factor*, VEGF), TNF-α, leptina, adiponectina, factor de crecimiento insulínico, interleuquina-6 (IL-6), factor activador del plasminógeno, resistina y angiotensinógeno. Muchos estudios han demostrado el papel significativo de PVAT como modulador de las contracciones del músculo liso vascular [17]. De hecho, si hablamos de patologías relacionadas con alteraciones de la función vascular como la hipertensión, hay estudios que explican cómo ésta se caracteriza por una reducción en el tamaño y cantidad de las células del PVAT, lo que se asocia con la disminución de su efecto anti-contráctil en los sujetos hipertensos [18]. Estudios recientes han demostrado que las enfermedades cardiovasculares y metabólicas alteran características morfológicas y funcionales del PVAT con notables consecuencias. En obesidad y diabetes, la expansión del PVAT contribuye a la aparición de resistencia a la insulina a nivel vascular y, a su vez, las citoquinas derivadas del PVAT pueden activar pasos claves del proceso de aterogénesis. Un denominador común de la disfunción del PVAT en estas condiciones patológicas es la infiltración de células del sistema inmune, lo que lleva a inflamación, estrés oxidativo y procesos de hipoxia que desembocan en la disfunción vascular [19].

Como se describirá más adelante, el **tejido adiposo** es un depósito dinámico de energía, el cual es responsable del almacenaje y movilización de la misma en

situaciones de déficit de nutrientes. En cambio, una excesiva ingesta de nutrientes produce una expansión en forma y tamaño de las células que conforman el tejido adiposo, lo que desemboca en una liberación de lípidos elevada y una producción descontrolada de adipoquinas, citoquinas y quimioquinas, teniendo lugar una serie de cascadas de señalización que conlleva en última instancia a un proceso inflamatorio en el tejido adiposo, considerado como un punto de inflexión en la obesidad y sus consecuencias (Fig. 3) [20]. Por otro lado, en esa expansión del tejido adiposo asociada a la obesidad tiene lugar un viraje también en el fenotipo de los macrófagos que abundan en el tejido graso, tendiendo el equilibrio de una producción mayoritaria de macrófagos anti-inflamatorios, como son los M2, a una sobreproducción de macrófagos pro-inflamatorios clásicos, de tipo M1. Este viraje en la población de macrófagos, unido al deterioro de la homeostasis de otros procesos celulares del sistema inmune, desemboca en una inflamación sistémica que participa activamente en la disminución de la sensibilidad a la insulina del tejido adiposo y de otros tejidos con función metabólica (Fig. 3) [20]. Es por esto que, un consumo excesivo de nutrientes por parte del individuo con sobrepeso puede agravar esta **inflamación** del tejido adiposo, lo que va a interferir en las acciones anabólicas y las rutas de señalización de la insulina con los tejidos correspondientes, causando una insulinorresistencia que va a traducirse en problemas de disponibilidad de glucosa para el músculo, y en un aumento de la lipolisis de los triglicéridos en el tejido adiposo, lo que dará lugar a la hipertrigliceridemia típica de la diabetes tipo 2 [3].

Fig. 3 Inflamación del tejido adiposo en el desarrollo de diabetes tipo 2 y Síndrome Metabólico [21]



Por último, podemos destacar otro de los hitos que conlleva el avance del proceso patológico del paciente obeso, y es la modificación del perfil lipídico, caracterizado por una hipertrigliceridemia que tras generar alteraciones en los niveles de HDL, LDL y VLDL da lugar a la formación de placas de ateroma. Como se ha indicado anteriormente, la resistencia a la insulina actúa como factor de riesgo cardiovascular independiente, pues además de intervenir en la formación de las placas de ateroma provoca la proliferación del músculo liso arterial. Esto a su vez influye en la aparición de disfunción endotelial e hipertensión, así como de **aterosclerosis**. Y es en este proceso aterosclerótico, el cual se origina por una acumulación de células inflamatorias en la capa íntima del vaso arterial, en el que se ve afectado de forma crítica una de las barreras más involucradas del organismo en cuanto a función vascular y que hemos descrito anteriormente: el endotelio [22].

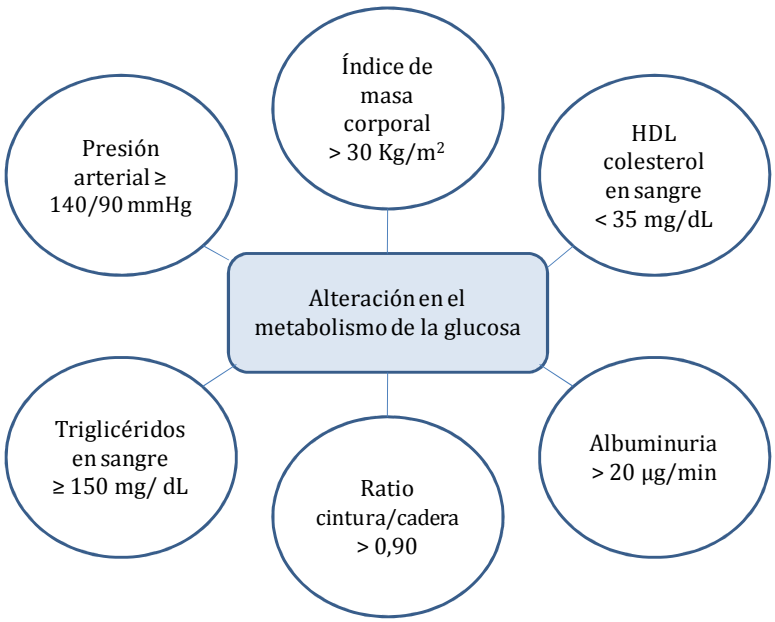
Como consecuencia de todos estos procesos, la hiperinsulinemia, hiperglucemia e hiperlipidemia que se generan como resultado de la resistencia a la insulina,

contribuyen en conjunto al desarrollo de diabetes tipo 2 y, finalmente, a la enfermedad cardiovascular. Por esta razón, el conjunto de estos desórdenes cardiometabólicos se consideran actualmente englobados dentro del concepto de **síndrome metabólico**. Se calcula que el 25 % de la población adulta del mundo puede llegar a padecer síndrome metabólico, el cual, como hemos descrito, se encuentra íntimamente ligado a la obesidad central o abdominal [23].

En la práctica, según la OMS, se considera síndrome metabólico a la coexistencia en un mismo individuo de diabetes o intolerancia a la glucosa junto con al menos dos de los siguientes criterios (Fig. 4):

- Índice de masa corporal superior a 30 kg/m^2 y/o cociente cintura-cadera superior a 0.90 en hombres o 0.85 en mujeres.
- Trigliceridemia mayor o igual a 150 mg/dL o niveles de HDL inferior a 35 mg/dL en hombres o 39 mg/dL en mujeres.
- Índice de excreción urinaria de albúmina superior a 20 µg/min.
- Presión arterial igual o superior a 140/90 mmHg.

Fig. 4 Condiciones patológicas del Síndrome Metabólico como factor de riesgo cardiovascular.



Según los criterios establecidos por la OMS, la coexistencia en un individuo de una alteración del metabolismo de la glucosa, como puede ser intolerancia a la glucosa, resistencia a la insulina o diabetes tipo 2, junto con dos o más de las condiciones patológicas indicadas en el esquema (círculos blancos), puede considerarse Síndrome Metabólico.

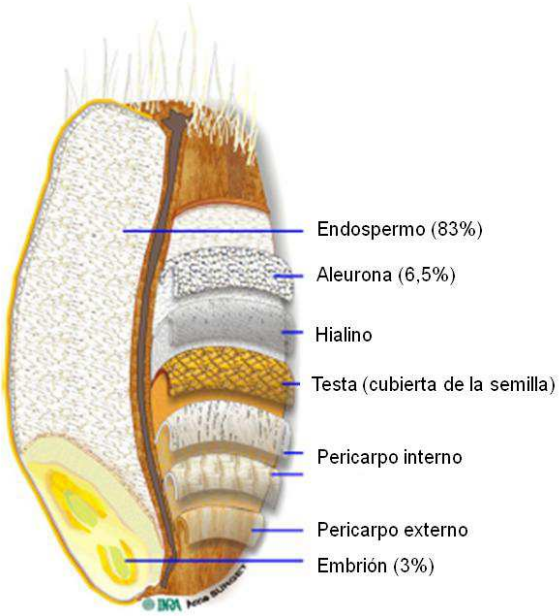
Los modelos animales, a pesar de ser imperfectos, nos han ayudado a descubrir que, aunque los mecanismos patológicos de la obesidad son complejos, incluyen un proceso inflamatorio que se cronificará en el tiempo y con el que hay que contar para aplicar una terapia eficaz. Sólo si entendemos los mecanismos o los puntos de unión mediante los que la obesidad fácilmente puede coincidir con otras patologías, entenderemos la naturaleza del síndrome metabólico dentro de los factores de riesgo cardiovascular. Si pensamos en estrategias de prevención, de los diez factores de riesgo identificados por la OMS como claves para el desarrollo de las enfermedades crónicas, cinco están estrechamente relacionados con la alimentación y el ejercicio físico, entre los que están la ya mencionada obesidad, el sedentarismo, la hipertensión arterial, la hipercolesterolemia y el consumo insuficiente de frutas y verduras [2]. En base a esto, parece interesante buscar estrategias nutricionales capaces de actuar sobre estas condiciones patológicas relacionadas con el síndrome metabólico y sus complicaciones.

2. POTENCIAL DEL SALVADO DE ARROZ Y SUS COMPONENTES BIOACTIVOS DENTRO DE LA TERAPIA NUTRICIONAL.

El arroz (*Oryza sativa*) constituye el alimento básico más importante para gran parte de la población mundial, sobre todo en el oriente Asiático, Latinomérica e India occidental. Se considera que el arroz proporciona una quinta parte del aporte de calorías consumidas en el mundo por los humanos. Se cultiva en al menos 114 países, con una producción global de 645 millones de toneladas, de las cuales, el 90 % es producido en Asia. El salvado de arroz, la capa marrón y más externa del grano de arroz, está compuesto principalmente por pericarpo, aleurona, sub-capa de aleurona y el germen (Fig. 5). El salvado contiene una importante cantidad de nutrientes como proteínas, grasas y fibra dietética. Además, contiene una cantidad notable de minerales como K, Ca, Mg y Fe [24]. También, su riqueza frente a otros

cereales en antioxidantes como son los compuestos fenólicos, flavonoides, antocianinas, proantocianidinas, tocoferoles, tocotrienoles y γ-oryzanol ha despertado el interés de este producto para mitigar ciertos problemas de salud. En cambio, su uso presenta ciertas limitaciones, ya que cuando el arroz es procesado a nivel industrial, hay ciertas dudas a la hora de reutilizar productos que se consideran "de desecho" como el salvado de arroz, el cual constituye un 5-8 % del total procesado. Normalmente, el salvado de arroz es considerado un producto obtenido tras el proceso de molienda del arroz y es utilizado para alimentación animal o descartado como material de desecho. El creciente interés por el aprovechamiento de estas materias primas se debe a que se trata de un producto infravalorado hasta ahora, el cual podría producirse a una escala de 29.3 millones de toneladas al año, y del que se han demostrado propiedades terapéuticas que beneficiarían a un amplio sector de la población de todo el mundo [26].

Fig 5 Estructura física del grano de arroz [25]

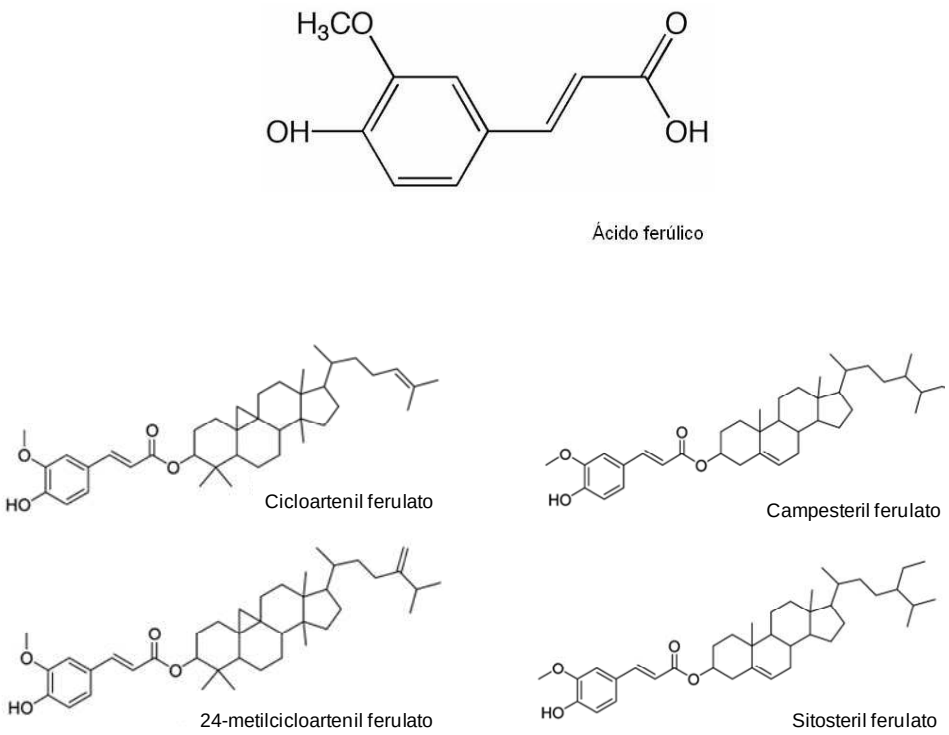


Si hablamos de la terapia que ha sido mayoritariamente aplicada frente las patologías que rodean a la obesidad, la dieta resulta ser la estrategia de primera línea en todos los casos. En los últimos tiempos, ha sido ampliamente demostrado que los granos integrales contienen combinaciones diversas de **fitoquímicos** (considerados moléculas o sustancias bioactivas de origen vegetal, que no tienen necesariamente una función nutritiva, pero que han demostrado reducir el riesgo de padecer enfermedades crónicas) según el tipo de cereal, el lugar del que provienen y de cómo

el grano ha sido procesado. La parte más externa de los granos contiene una cantidad mucho más elevada de fitoquímicos (compuestos fenólicos, fitosteroles, tocoles, betaína y folato) que el germen y el endospermo [25]. Los **compuestos fenólicos** constituyen el grupo de fitoquímicos más complejo y diverso dentro de los granos de los cereales. Entre ellos se incluyen numerosos derivados del ácido benzoico y cinámico tales como flavonoides, flavonas y flavanoles, antocianidinas, avenantramidas, lignanos y alquilresorcinoles. En la mayoría de los granos, los ácidos fenólicos se encuentran concentrados en el salvado y en las paredes de las células embrionarias, y están mayormente en su forma insoluble, libre o en forma conjugada soluble, aunque esto es en el menor de los casos [27]. Por otro lado, el grano de arroz en general supone también una fuente importante de **antioxidantes liposolubles** como los fitosteroles combinados con ácido ferúlico (γ-oryzanol), tocoferoles y tocotrienoles, aunque el contenido en estos micronutrientes varíe bastante según las variedades de arroz. En cambio, lo que sí se ha constatado es el elevado contenido en derivados del ácido ferúlico que contienen los arroces integrales, los cuales en comparación con mezclas de arroz blanco, basmati y silvestre, se ven aumentados hasta 3 veces más (Fig. 6). Se ha observado que existe una relación directa entre el contenido fenólico total de estos arroces y su capacidad antioxidante, probada en numerosos ensayos *in vitro*, la cual es 30 veces mayor en el caso del arroz silvestre que en el arroz blanco [28]. Por otro lado, ensayos clínicos han demostrado cómo una dieta saludable donde participen alimentos a base de arroz integral puede tener beneficios en pacientes con síndrome metabólico, disminuyendo en ellos el riesgo de sufrir enfermedades cardiovasculares [29]. Según estudios como los de Fabian & col., el salvado de arroz ha sido utilizado en forma de extractos para la experimentación de diferentes maneras: preservando su contenido graso, eliminándolo, en forma de aceite y como concentrados de proteínas. En general, el salvado contiene entre un 10-15 % de lo que se ha definido como **proteínas de alta calidad**. Hay autores que afirman que, aunque parece inviable su

comercialización, las proteínas del salvado comparadas con las de otros cereales presentan propiedades hipoalergénicas y antitumorales exclusivas [30].

Fig. 6 Estructuras químicas de las moléculas más relevantes del γ-oryzanol



De forma rutinaria, y con el fin de aportarles propiedades para el paladar, los granos de arroz pasan por varias etapas como descascarillado, molienda y deshidratación. Aunque estos tratamientos pueden reducir el contenido fitoquímico, la biodisponibilidad de éstos a menudo se ve incrementada. El tratamiento térmico y el bio-procesamiento también pueden mejorar la biodisponibilidad de los componentes, principalmente el procesamiento mediante medios biológicos, aunque los resultados no sean fácilmente consistentes o reproducibles. Por tanto, el **método de extracción y procesamiento** del arroz va a ser otro paso determinante en las propiedades del extracto [31, 32]. Hay estudios que afirman en este sentido que los productos derivados del arroz que han sufrido un tratamiento por radiación infra-

roja de larga distancia acaban teniendo un mayor contenido en compuestos bioactivos como los tocoferoles y algunos otros compuestos fenólicos y, por tanto, preservan mejor sus propiedades antioxidantes que aquellos que son deshidratados con corriente de aire caliente y celulasas. En cambio, en otros estudios como los realizados por Anson & col., se han obtenido notables resultados en cuanto al mantenimiento de la integridad de las propiedades de otros extractos mediante la fermentación y extracción enzimática posterior. En el caso del extracto de salvado de arroz que se utiliza **en nuestro estudio**, un extracto enzimático del salvado de arroz (EESA), se le han atribuido una serie de beneficios en base a su composición, rica en compuestos bioactivos de diferente naturaleza como son ácidos grasos insaturados, alcoholes triterpénicos, fitosteroles, tocotrienoles y tocoferoles, γ-oryzanol y vitamina E [33].

Cuando hablamos de **biodisponibilidad**, nos referimos a la fracción de fitoquímicos ingeridos (u otros compuestos dietéticos) que alcanza la circulación sistémica. De forma más común, se define como la fracción que es absorbida en el tracto gastrointestinal. Métodos de marcaje molecular, en los que átomos o moléculas de los fitoquímicos del arroz son rastreados con algún marcador intrínseco radiactivo, supone de momento la única manera de determinar la biodisponibilidad real de una sustancia [28]. Dada la dificultad que supone el marcar fitoquímicos en el caso de los granos de arroz, se ha recurrido a métodos indirectos para determinar la biodisponibilidad como puede ser el balance de lo ingerido menos lo eliminado de forma fecal, o el incremento del área bajo la curva de la concentración postprandial en suero respecto al incremento de la excreción vía urinaria. Son varios los estudios que afirman que la biodisponibilidad de los compuestos del arroz es difícil de determinar a nivel del intestino delgado, aunque se ha observado que aumenta en el caso del salvado de arroz por ser la parte más rica en fitonutrientes, y a su vez, ésta se ve incrementada en el caso de los compuestos liposolubles como son los ferulatos [28]. Como hemos indicado anteriormente, hay autores que han demostrado que los

alimentos constituidos a base de cereales y que son bioprocesados ven incrementada de forma remarcable la biodisponibilidad de los ácidos fenólicos y sus metabolitos circulantes, compuestos que presentan efectos inmunomoduladores en experiencias *ex vivo*. Hay estudios donde la biodisponibilidad de compuestos como el ácido ferúlico, vaníllico, sinápico y 3,4-dimetoxibenzoico procedentes de pan bioprocesado fue de 2 a 3 veces mayor que la del pan original [34].

Algo que no debemos olvidar si hablamos de los fitonutrientes que son finalmente asimilados, es que la parte más externa del grano, el salvado, es rico en **ácido ferúlico** (AF); sin embargo, su biodisponibilidad o su paso a través del intestino desde la matriz es muy baja. Esta disminuida biodisponibilidad se explica por la estructura de la mayoría de los derivados del AF (Fig. 6), ya que se trata de una molécula que tiende a formar enlaces covalentes no absorbibles por la pared celular, constituyendo la fibra dietética o insoluble [35]. A nivel del intestino grueso, donde la microbiota del individuo juega su papel, hay estudios que ponen de manifiesto la ventaja del AF que es ingerido por encontrarse en el grano de arroz frente al que es administrado como compuesto puro, ya que como se ha indicado antes, los extractos naturales con este tipo de composición ejercen su función de forma sinérgica, y en el caso del salvado de arroz es la misma fibra la que va a favorecer la absorción y biodisponibilidad de los compuestos como el AF gracias a los procesos que sufre frente a la microbiota del intestino. De hecho, se ha demostrado en animales cómo la actividad antioxidante del AF en plasma es significativamente mayor si proviene del arroz integral que si se trata del compuesto puro [36]. En cambio, varios autores han demostrado que el posible paso a nivel sistémico de estos componentes tiene una corta duración, la cual ronda entre las 6-24 h tras la ingesta. El hecho de que este tiempo sea tan variable se debe a la acción precisamente de la microbiota colónica, la cual puede favorecer en un primer momento la llegada de sustancias a la sangre pero las hace muy lábiles [37].

Con la idea de preservar la integridad de los componentes más destacables del salvado de arroz, entre los que podemos incluir como hemos dicho los derivados del AF, fitosteroles, tocoferoles y tocotrienoles, la mayoría de los estudios que se han realizado para probar la actividad farmacoterapéutica han utilizado **extractos oleosos** de salvado. De esta manera, y por la naturaleza liposoluble de estos componentes, el extracto mantiene sus propiedades intactas, pero aparecen limitaciones en relación a su conservación a medio y largo plazo, ya que presentan problemas de enranciamiento y pérdida de propiedades organolépticas [9, 38].

En este sentido, resulta necesario encontrar un procesamiento del salvado de arroz que permita concentrar, hidrolizar y estabilizar sus componentes con la idea de salvar todos estos obstáculos. Precisamente en esta línea es donde se propone un nuevo método de extracción enzimática mediante el que se obtiene **EESA**, un extracto en forma de sirope color marrón que presenta una serie de ventajas frente al salvado de arroz natural: es hidrosoluble, lo que le aporta facilidades a nivel de conservación, manipulación y administración frente a otros extractos; contiene un elevado contenido en proteínas de un importante valor nutricional (38 %) y fácilmente asimilables por el organismo debido a su bajo peso molecular (<10 kDa); es rico en compuestos liposolubles que gracias a la composición proteica del extracto no muestran problemas de solubilidad, e incluye ácidos grasos poliinsaturados, vitamina E y 3,4 veces más cantidad de γ-oryzanol que el salvado de arroz natural [39].

Debido al interés que ha despertado el salvado de arroz como posible complemento nutricional debido a su composición, rica en nutrientes con potencial terapéutico, se han descrito diversas actividades beneficiosas frente a varias de las condiciones patológicas que se asocian con el síndrome metabólico (Tabla 1):

2.1. Actividad antihipertensiva

En los últimos años ha sido ampliamente estudiada la asociación inversa que existe entre el riesgo de padecer enfermedades cardiovasculares y el consumo de alimentos y/o bebidas ricas en **polifenoles** como son el cacao, las frutas, las verduras, el té, el aceite de oliva virgen o el vino. En el caso de los polifenoles, se han obtenido notables resultados en cuanto a biodisponibilidad siempre que son ingeridos en los alimentos. A su vez, sus beneficios sobre los niveles de presión arterial han sido relacionados con su capacidad para aumentar la síntesis de NO y la respuesta mediada por el EDHF [40]. Como se ha estudiado ampliamente, la disfunción endotelial y la hipertensión están íntimamente relacionadas con la obesidad y la resistencia a la insulina. Por tanto, todo compuesto nutricional o extracto alimenticio que sea capaz de actuar sobre las alteraciones vasculares asociadas a la obesidad son susceptibles de ser utilizados en su prevención. Aunque haya casos en los que la disfunción vascular en animales obesos no es tan patente, sí se ha visto que fracciones o productos ricos en polifenoles son capaces de preservar la capacidad vasorelajante de las arterias cuando la liberación de NO y de los derivados de la enzima ciclooxigenasa (COX) se encuentran inhibidas, teniendo efectos sobre otros mecanismos como la respuesta mediada por el EDHF [41].

Autores como Shirakawa & col. (2007), basándose en la idea de que cualquier compuesto alimenticio que fuera capaz de actuar sobre la presión arterial podía funcionar en la prevención de las enfermedades cardiovasculares, han estado trabajando con un extracto de salvado de arroz tratado con la enzima driselasa (Tabla 1). En un principio, quisieron comparar los efectos en animales que podía tener la administración del extracto y de uno de sus compuestos mayoritarios aislado, el **AF**, sobre la hipertensión y el metabolismo de la glucosa y los lípidos. Como resultado, llegaron a la conclusión de que, aunque ambos tratamientos presentaban efectos beneficiosos, el suplemento en la dieta con el extracto de

salvado de arroz tratado con driselasa presentaba numerosos efectos protectores frente a la hipertensión, dislipemias e hiperglucemia desarrollada por las ratas tratadas, y de forma más pronunciada que en el caso del AF aplicado como compuesto puro.

En cambio, en la búsqueda de aportar algún dato determinante en cuanto al compuesto que podría ser responsable de la descrita acción hipotensora del salvado de arroz, los autores proponen también a la **adenosina**, uno de los componentes presentes en el mismo extracto, y su administración oral aguda y crónica, como compuesto capaz de mejorar de forma significativa parámetros como la presión arterial, y triglicéridos, glucosa y NO en plasma a las 2 h después de su administración a ratas hipertensas. Por tanto, concluyen que esta sustancia podría ser activa frente a los síntomas patológicos asociados al síndrome metabólico [42]. Otros autores han confirmado en la actualidad los beneficios del AF, capaz de mejorar la relajación dependiente de endotelio en aorta de rata y el estado de oxidación en diferentes órganos, ya que es capaz de incrementar la actividad de la superóxido dismutasa y la catalasa en el corazón y en los riñones. Por otro lado, disminuyó la actividad enzimática en hígado y plasma, además de la concentración de creatinina. A la luz de estos resultados, proponen al AF como una molécula capaz de mejorar las alteraciones funcionales y estructurales de corazón, vasos sanguíneos, hígado y riñones en animales hipertensos [43].

En base a los estudios descritos, y teniendo en cuenta la riqueza en polifenoles y derivados del AF (γ-oryzanol) que presenta el extracto utilizado en este estudio (EESA), cabría esperar que su administración indujera cierta restauración de los niveles de presión arterial en animales que hubieran desarrollado hipertensión moderada. Por otro lado, debido a la importancia del NO como principal agente vasodilatador, y viéndose su biodisponibilidad comprometida en las patologías incluidas en el síndrome metabólico, también pensamos que resultaría interesante probar los efectos de **EESA** en las arterias debido a su riqueza en

Arginina, aminoácido precursor de la síntesis de NO y destacable dentro de la composición del extracto, suponiendo casi un 13 % de su contenido proteico [44].

2.2. Actividad sobre el metabolismo de la glucosa

Multitud de experiencias realizadas con diferentes **alimentos refinados e integrales** han permitido concluir que, aunque serían necesarios más estudios con alimentos no refinados para confirmarlo, sí es cierto que el elevado consumo de alimentos refinados es directamente proporcional al incremento de riesgo de padecer diabetes tipo 2. En cambio, se ha demostrado que consumir de 2 a 3 raciones al día de alimentos integrales disminuye casi al 30 % el riesgo de desarrollar diabetes tipo 2 [45].

El grano integral contiene como hemos dicho, el endospermo, el germen y el salvado, cosa que lo diferencia del grano refinado, que ha perdido toda la capa externa en el proceso de molienda y está constituido sólo por el endospermo [28]. La capacidad del grano integral de disminuir el riesgo de diabetes tipo 2 es atribuida a su contenido en fibra, vitaminas, minerales y sustancias fitoquímicas que pueden mejorar la sensibilidad a la insulina y actuar sobre el metabolismo de la glucosa, mediante una mejora del sobrepeso y la obesidad [46]. La mayoría de los estudios observacionales concluyen que **la fibra insoluble** ejerce un papel protector mayor que la fibra soluble, siendo la más característica en las comidas integrales. En cambio, aunque podemos encontrar en los granos integrales una amplia gama de micronutrientes, resulta muy difícil relacionar sus efectos beneficiosos con uno sólo de sus componentes, como puede ser el caso de la fibra [24].

Por otro lado, y como se apuntaba anteriormente, la hiperlipidemia y la hiperglucemia que acompañan al deterioro funcional de las células β-pancreáticas, y por tanto, afectan a la acción de la insulina, provocan un grado de

estrés oxidativo o una elevada producción de radicales libres que va a propagarse en el sujeto diabético a nivel sistémico, afectando a diferentes órganos diana [47, 48]. Por tanto, los compuestos derivados de la dieta como son los fitoquímicos y un grupo concreto de micronutrientes con poder antioxidante, pueden prevenir el desarrollo y progresión del síndrome metabólico y diabetes tipo 2 mediante la reducción de estrés oxidativo. En este aspecto, adquiere interés el estudio de las propiedades farmacoterapeúticas que puede tener la administración de **EESA** sobre animales con alteraciones en el metabolismo de la glucosa, ya que la presencia entre sus componentes de tocoferoles y tocotrienoles, sobre todo en su conformación como α-tocoferoles y α-tocotrienoles, le aporta parte de su actividad antioxidante junto con su elevada cantidad de vitaminas y minerales [44]. De hecho, la propiedad de **EESA** en la que más se ha profundizado desde los primeros ensayos realizados por Parrado & col. en 2003 ha sido en su capacidad antioxidante, con idea de demostrar la aplicación que podía tener en el ámbito de las enfermedades crónicas que cursan con un elevado estrés oxidativo [49].

2.3. Actividad frente a las dislipemias

La hiperlipidemia es un problema global que puede tener consecuencias como una elevada presión arterial, elevados niveles de glucemia, enfermedad coronaria, daño hepático, y todo esto puede agravarse por la edad. Cuando hablamos de lípidos en el organismo, nos referimos principalmente al colesterol total y triglicéridos (TG) circulantes. Las lipoproteínas incluyen las VLDL-c, LDL-c y HDL-c, y las hiperlipidemias se generan cuando hay una producción excesiva de VLDL-c o una conversión excesiva de LDL-c desde las VLDL-c. Por tanto, controlar la hipercolesterolemia se considera la clave para actuar sobre las hiperlipidemias [50]. Varios autores han demostrado que al ser ricos los aceites de salvado de arroz en **fitosteroles**, estas moléculas actúan inhibiendo la

absorción del colesterol en el intestino, interfiriendo en la formación de micelas. Además, los fitosteroles también actúan sobre el líquido biliar, cuya síntesis está íntimamente relacionada con la del colesterol. Se ha visto que hay, dentro de estas moléculas, algunas más efectivas en la reducción de la absorción del colesterol por similitud estructural, como es el 4-desmetilesterol. Una de las ventajas de esta sustancia y sus derivados es que, por su estructura química, son absorbidas más fácilmente por el intestino que el mismo colesterol [51, 52]. Por otro lado, se ha visto que algunos componentes característicos de los salvados de arroz más pigmentados, como la **antocianina**, es muy efectiva en la inhibición de la oxidación del colesterol y menos en la de los ácidos grasos. Los estudios en humanos sugieren que el salvado de arroz, sus extractos y sus compuestos bioactivos, parecen ejercer su acción anti-hipercolesterolémica de forma más efectiva en individuos con dislipemias que en aquellos con un perfil lipídico normal en suero [30]. De hecho, se ha visto que el aceite de salvado mejora el perfil lipídico, el riesgo de aterogénesis y los factores de riesgo cardiovascular en pacientes hiperlipidémicos de forma efectiva [53, 54].

También hay estudios donde se destaca el efecto hipo-colesterolémico del compuesto quizás más destacable del salvado de arroz, el **γ-oryzanol**, una macro-molécula formada por ésteres de ácido ferúlico y fitosteroles llamados fitosteril ferulatos, capaz de reducir en plasma el colesterol LDL y VLDL, y aumentar el HDL mediante la estimulación de la secreción de colesterol y sus metabolitos, ejerciendo así su actividad hipocolesterolémica [33, 55] . Además, se ha observado que es capaz de producir un incremento de la actividad de enzimas antioxidantes hepáticas y mejorar del índice aterogénico LDL-c/HDL-c [56]. En base a estos datos, la actividad hipocolesterolémica del γ-oryzanol ha sido probada tanto en animales como en humanos, y se le ha atribuido diferentes mecanismos de acción, entre los que se incluye la posible interferencia que puede ejercer el γ-oryzanol en la incorporación del colesterol a las micelas durante su

paso por el intestino delgado, en detrimento de la transferencia del colesterol desde las micelas a los enterocitos; la influencia derivada de la similar estructura química de los componentes del γ-oryzanol y el colesterol; el incremento como hemos dicho anteriormente de la excreción fecal de ácidos biliares y esteroles neutros; el aumento de la expresión de enzimas como la CYP7A1, clave en la descomposición del colesterol; y la inhibición de la actividad de la enzima hidroximetilglutaril coenzima A (HMG-CoA) reductasa, enzima limitante de la biosíntesis del colesterol [57].

Los ensayos clínicos demuestran que una ración diaria de 300 mg de γ-oryzanol disminuye los niveles de colesterolemia en los pacientes. Lo que nos lleva a pensar que **EESA** podría ser un buen candidato a ensayar en pacientes debido a su riqueza en esta sustancia, la cual contiene casi por triplicado en comparación con el salvado de arroz de partida [39].

2.4. Actividad anti-inflamatoria

Los mecanismos celulares y moleculares implicados en el proceso inflamatorio son diversos, y hay un gran número de mediadores cuya función ya ha sido bien descrita, como el factor activador plaquetario, prostaglandinas, leucotrienos, aminas, purinas, citoquinas, quimioquinas y moléculas de adhesión. Todos estos mediadores son secretados por los leucocitos para atraer a otras células, como los neutrófilos, al lugar de la inflamación. Recientemente, se ha descubierto el nexo de unión entre el proceso inflamatorio y numerosas enfermedades crónicas como el Alzheimer, la aterosclerosis o el cáncer. En este sentido, y con el objetivo de buscar nuevas estrategias terapéuticas, han cobrado valor por su actividad reguladora de la inflamación muchos productos naturales [58]. Existe una amplia bibliografía sobre las propiedades antiinflamatorias de los fitoquímicos derivados de los cereales, y concretamente del grano integral [59, 60], lo que convierte a este producto en una posible herramienta nutricional para enfrentar

ese estado de inflamación crónica asociada con el balance de estrés oxidativo/nitrosativo, ratio que va a marcar la vulnerabilidad del material genético ante los agentes pro-oxidantes, delecciones y mutaciones que pueden dar lugar a la carcinogénesis [61].

Los **polifenoles** de la dieta han sido ampliamente estudiados por multitud de aplicaciones, pero una de las acciones que más interés ha despertado en los últimos años son los mecanismos por los que pueden ejercer su acción como moduladores de la respuesta inmunitaria [30]. Son varias las sustancias fitoquímicas que actúan en la supresión de la activación de factores de transcripción como el NF-κβ, presentando actividad antialérgica y anti-inflamatoria. Se ha demostrado que el γ-oryzanol es capaz de capturar la IgE, evitando que tenga lugar la reacción alérgica en la piel de ratas, evitando la unión covalente con el receptor FceR1 y atenuando la desgranulación de mastocitos. Se ha observado que este compuesto es capaz de actuar como anti-alérgico en la reacción anafiláctica cutánea pasiva. También existen preparados como el BioBran®, un arabinoxilano modificado enzimáticamente procedente del salvado de arroz, el cual, es un potente acelerador de la maduración de células dendríticas, lo que lo convierte en un estimulante del sistema inmunitario, como también se ha observado en el caso de **EESA** gracias a su contenido en arabinoxilano y, por supuesto, su riqueza en γ-oryzanol [62]. Uno de los mecanismos anti-inflamatorios más aceptados para el γ-oryzanol como uno de los componentes más representativos del salvado de arroz, ha sido el de actuar como supresor de la activación del NF-κβ, inhibiendo la respuesta inflamatoria producida por macrófagos e incrementando la secreción de adiponectina por parte de los adipocitos [63]. A su vez, este mecanismo ha demostrado ser eficaz frente a la sobreexpresión de las moléculas de adhesión ICAM y VCAM inducida por lipopolisacárido (LPS) en el endotelio vascular [64].

2.5. Otros efectos beneficiosos descritos:

2.5.1. Actividad antitumoral

El proceso canceroso se caracteriza por la desregulación de múltiples genes, lo que da lugar a una serie de síntomas adversos. Entre otros, se ven afectados genes relacionados con la expresión de NF-κβ o la IL-6, mediadores muy involucrados en el cáncer. Se ha visto que hay compuestos incluidos en la dieta que actúan sobre la activación del NF-κβ y procesos paralelos que pueden aliviar el proceso tumoral. Se han realizado estudios en animales donde se demuestra que el salvado de arroz y sus compuestos bioactivos inhibían la carcinogénesis hepática, esofágica, gástrica y en el colon. En los ensayos *in vitro*, diferentes extractos de salvado de arroz han demostrado efectos positivos sobre las siguientes líneas celulares cancerosas: endometrio, leucemia, mama, colon, hígado, melanoma, páncreas, ovario y cáncer colorrectal [30]. Por ejemplo, se ha descrito que el γ-oryzanol resulta efectivo frente a los procesos tumorales si se aplica al 0,2 % en la dieta, siendo capaz de inducir la actividad de las células NK (*Natural Killers*), la activación de macrófagos e inhibiendo el proceso de angiogénesis dentro del tejido tumoral [65]. Por otro lado, el uso de ciertos **alimentos integrales** (granos, fruta, legumbres, té o especias) se han asociado a una disminución del riesgo de cáncer, y su seguridad inherente convierte a estos posibles alimentos funcionales en una opción interesante para promover su uso de forma general y durante tiempos prolongados en diferentes grupos de población de alto riesgo [66].

En resumen, se ha concluido que el mecanismo por el que los componentes bioactivos que se encuentran de forma exclusiva en el salvado de arroz realizan su acción quimiopreventiva tiene cuatro puntos de actuación: (1) su actividad antioxidante, definida por su acción sobre diferentes enzimas diana del organismo involucradas en la defensa frente al estrés oxidativo; (2) su actividad anti-proliferativa y pro-apoptótica, capaz de regular posibles procesos

descontrolados del crecimiento celular (como ya se ha demostrado en el caso de **EESA** con Parrado *et al.*,[39] ; (3) su actividad inmunomoduladora, donde actúa sobre diferentes genes y moléculas clave en el proceso inflamatorio y en la respuesta inmune; (4) su efecto protector sobre la mucosa intestinal y colónica, preservando la función de la microbiota, pero limitando una excesiva actividad metabólica y enzimática que altere las condiciones de la pared celular, alterándose su permeabilidad y el equilibrio en el transporte de sustancias a nivel sistémico.

2.5.2. Actividad cosmecéutica

Como hemos descrito anteriormente, son varios los autores que ponen de manifiesto el papel que juega una dieta rica en granos integrales en la reducción de la oxidación de lípidos a nivel plasmático y urinario. Por otro lado, la defensa antioxidante que genera una dieta rica en granos íntegros se ha probado sobre todo en la mejora del nivel antioxidante en sangre mediante la modulación del sistema antirradicalario del **glutatión**. En este sistema, la glutatión peroxidasa hidroliza peróxido de hidrógeno utilizando el glutatión reducido como donador de hidrógeno. Si bien es cierto que hay evidencias firmes sobre el poder antioxidante de los compuestos del salvado de arroz, aún existen dudas acerca del tipo de arroz y el tiempo de consumo óptimo para obtener ese efecto regulador de la enzima glutatión y su estado redox [28, 67]. Sin embargo, el **EESA** cuyas propiedades presentamos en este estudio presenta un contenido relativamente alto de los aminoácidos azufrados cisteína y metionina (6 % de las proteínas totales), los cuales desempeñan un importante papel en la acción antioxidante de **EESA**, ya que actúan como precursores del glutatión y la taurina (antioxidantes naturales) y, además, su grupo SH participa directamente en la reacciones redox [44].

Debido a esta cualidad generalizada en sus componentes, el aceite de salvado de arroz ha despertado un elevado interés en la fabricación de formulaciones

farmacéuticas y cosméticas [50]. Hay autores que han elaborado formulaciones de distinta naturaleza con extractos de salvado de arroz ricos en ɣ-oryzanol, ferulatos, ácido fítico recogidos en niosomas y con la finalidad de ser utilizados frente al envejecimiento de la piel. Estas formulaciones no sólo presentan capacidad de estimular la formación de fibroblastos en humanos y de inhibir la actividad MMP-2, sino que han demostrado producir mejoras en la piel a nivel de hidratación, pigmentación, firmeza, rugosidad y elasticidad [68-70]. De hecho, se han probado las propiedades fotoprotectoras de **EESA** con el objetivo de determinar las aplicaciones que podría tener en el campo de la dermatología [71].

TIPO DE EXTRACTO DE SALVADO DE ARROZ		COMPUESTOS DESTACABLES	ACTIVIDAD TERAPÉUTICA	REFERENCIA
OLEOSOS	Aceite	Fitosteroles AG poli-insaturados	Reducción de la actividad HMG-CoA	[72] (A)
	Aceite	ɣ-oryzanol	Disminución de colesterol total, LDL-c y LDL-c/HDL-c	[73] (H)
	Nanoemulsión	Composición general	Mejora de patologías de la piel como psoriasis o dermatitis	[70] (H)
	Aceite	ɣ-oryzanol ɣ -tocotrienol	Reducción de la absorción de colesterol e incremento de receptores LDL	[51] (A)
	Aceite	ɣ -oryzanol ɣ-tocotrienol	Mejora del perfil lipídico, índice aterogénico e hiperinsulinemia en diabetes tipo 2	[52] (A)
	Estabilizado	ɣ-oryzanol	Efectos frente al deterioro neurodegenerativo	[74] (A)
			Efectos frente a la diabetes	[47] (A)
	Aceite	Enriquecido con liquido de mantenimiento	Mejora de la calidad del semen congelado de jabalí	[75] (A)
	Aceite	ɣ-oryzanol, Fitosteroles Tocotrienoles	Disminución de colesterol y TG en suero	[76] (H)
	Aceite	ɣ-oryzanol Fitosteroles	Disminución de los niveles de LDL en pacientes hiperlipidémicos	[77] (H)
	Aceite	α-tocotrienol ɣ-tocotrienol ɣ-oryzanol	Efecto anti-hipercolesterolémico	[35] (H)
	Nanopartículas	Composición de origen	Actividad antioxidante y fotoprotectora	[78] (A)

Tabla 1. Recopilación de estudios realizados con extractos de salvado de arroz desde el año 2000.

TIPO DE EXTRACTO DE SALVADO DE ARROZ		COMPUESTOS DESTACABLES	ACTIVIDAD TERAPÉUTICA	REFERENCIA
OLEOSOS	Aceite	γ-oryzanol γ-tocotrienol γ-tocoferol	Disminución del estrés oxidativo y mejora de la estructura de los tejidos en diabetes tipo 2	[48] (A)
	Enriquecido	DAG	Disminución de los niveles de colesterol y TG en plasma	[79] (A)
	Aceite	γ-oryzanol y otros compuestos antioxidantes	Retraso de la carcinogénesis colónica inducida con DSS	[38] (A)
	Aceite	Tocotrienoles	Prevención de la nefropatía diabética	[9] (A)
	Aceite	Composición de AG	Disminución del índice aterogénico	[54] (H)
	Aceite	γ-oryzanol Tocoferoles	Reducción de lipoproteínas no-HDL e incremento de HDL en plasma	[55] (A)
	Aceite	γ-oryzanol	Mejora de factores de riesgo CV en pacientes hiperlipidémicos	[53] (H)
LIOFILIZADOS	Extracto etanólico y tratado con Driselasa	Composición general	Mejora de la presión arterial, metabolismo de la glucosa y perfil lipídico en ratas hipertensas	[36] (A)
	Tratado con Driselasa	Adenosina	Atenúa SM en ratas hipertensas	[42] (A)
	Tratado con CO_2	Ácido linolénico γ-tocotrienol γ-oryzanol	Actividad frente a la pérdida del cabello	[80] (A)

Tabla 1. Recopilación de estudios realizados con extractos de salvado de arroz desde el año 2000. (Continuación)

TIPO DE EXTRACTO DE SALVADO DE ARROZ		COMPUESTOS DESTACABLES	ACTIVIDAD TERAPÉUTICA	REFERENCIA
LIOFILIZADOS	Exo-biopolímero	Arabinoxilano	Aumento de IFN- ϒ sin efectos adversos. Actúa como inmunoestimulante.	[114] (H)
	Liofilizado	Proteínas	Regeneración de células osteogénicas	[81] (A)
	Extracto metanólico	ɣ-oryzanol Compuestos fenólicos	Actividad antioxidante	[67] (A)
	Niosomas para uso tópico	Ácido ferúlico ɣ-oryzanol Ácido fítico	Efecto anti-edad	[68] (A)
	Disolución	---	Inhibición de la proliferación de células cancerosas del colon	[82] (A)
ENZIMÁTICOS	Extracto Enzimático de Salvado de Arroz (EESA)	ϒ-oryzanol Fitosteroles Proteínas	Prevención de enfermedades crónicas relacionadas con elevado estrés oxidativo	[49] (A)
			Efecto anti-proliferativo	[39] (A)
			Efecto anti-hipercolesterolémico y antioxidante	[33] (A)
			Efecto anti-radicalario. Protección UVB	[71] (A)
			Efecto anti-proliferativo e inmunoestimulante	[62] (A)

Tabla 1. Recopilación de estudios realizados con extractos de salvado de arroz desde el año 2000. (Continuación)

(H): ensayos clínicos; (A): estudios en animales; AG: Ácidos grasos; DAG: diacil-glicerol; TG: triglicéridos; CV: cardiovascular; SM: síndrome metabólico; IFN-ɣ: interferón-gamma; UVB: ultravioleta B

JUSTIFICACIÓN Y OBJETIVOS

El salvado de arroz es un sub-producto obtenido tras el procesamiento del arroz integral, del cual, a pesar de haber sido infrautilizado por su difícil extracción, conservación y administración, se han descrito múltiples beneficios para la salud atribuibles a su composición, rica en compuestos bioactivos. Por tanto, parece interesante estudiar los efectos del salvado de arroz sobre uno de los ámbitos que más afectan a la salud en nuestros días, como es la obesidad y el Síndrome Metabólico.

La realización de esta tesis tuvo como objetivo general el estudio de las propiedades beneficiosas que la administración de un extracto enzimático de salvado de arroz (EESA) puede tener frente a las complicaciones vasculares y metabólicas que se asocian al desarrollo del Síndrome Metabólico como factor de riesgo cardiovascular en modelos animales de obesidad.

Con esta finalidad, se establecieron como objetivos específicos, determinar:

- Si la administración en la dieta de un suplemento de EESA (al 1% y 5%) es capaz de atenuar alteraciones de la presión arterial, del metabolismo de la glucosa y del perfil bioquímico asociadas al Síndrome Metabólico en ratas Zucker obesas.

- Si una dieta suplementada con EESA tiene efectos sobre la disfunción endotelial y vascular que se aprecia en la aorta de ratas Zucker obesas. Además, se estudiarían los mecanismos moleculares involucrados en dichos efectos, sobre todo, los relacionados con el estrés oxidativo y la inflamación vascular.

- Si EESA es capaz de corregir alteraciones de la microvasculatura de ratas Zucker obesas, por ser uno de los factores de riesgo cardiovascular detectables de forma temprana. Se estudiaría de forma especial la producción de NO, EDHF y anión superóxido en los vasos de resistencia de los animales obesos tratados con EESA.

- Si el tratamiento con EESA es capaz de minimizar las modificaciones morfológicas y funcionales del tejido adiposo, actuando sobre la dinámica molecular pro-inflamatoria característica del Síndrome Metabólico desarrollado en el modelo Zucker.

- Si EESA tiene actividad en un modelo de obesidad inducida por la dieta en ratones, enfocándonos en los beneficios que puede aportar frente a las alteraciones del metabolismo de la glucosa, de los parámetros bioquímicos y de la morfología y funcionalidad del tejido adiposo. A su vez, se determinaría el efecto de EESA sobre el fenotipo de los macrófagos y sobre la expresión de genes pro-inflamatorios en el tejido adiposo blanco.

ARTÍCULO 1: **Water-soluble rice bran enzymatic extract attenuates dyslipidemia, hypertension and insulin resistance in obese Zucker rats**

European Journal of Nutrition (2013)

Un extracto enzimático de salvado de arroz soluble en agua atenúa la dislipemia, la hipertensión y la resistencia a la insulina en ratas Zucker obesas

Justo *et al,* 2013. *European Journal of Nutrition*

El salvado de arroz es un residuo orgánico que se obtiene tras el proceso de molienda del arroz, y procede de la capa más externa del grano. A pesar de su demostrado potencial como alimento funcional, su uso se ha visto limitado por la insolubilidad que le aporta su elevado contenido proteico, por la dificultad a la hora de mantener la integridad de sus componentes tras los procesos de extracción, y en el caso de los extractos oleosos, por los problemas de enranciamiento y la difícil administración de los mismos [24]. Gracias a la optimización de los métodos de extracción, el grupo de investigación de Tecnología de la Producción de Enzimas del Departamento de Bioquímica y Biología Molecular de la Facultad de Farmacia, ha logrado producir un extracto enzimático de salvado de arroz (EESA) con importantes ventajas sobre el resto de extractos estudiados hasta la actualidad, entre ellas, su hidrosolubilidad, su elevado contenido en proteínas de alto valor nutracéutico y su riqueza tres veces mayor en γ-oryzanol que el salvado de arroz de partida [39]. El objetivo de nuestro estudio fue determinar los efectos beneficiosos de EESA sobre el síndrome metabólico desarrollado en ratas Zucker obesas.

Para ello, durante 20 semanas se les administró una dieta estándar enriquecida al 1% y 5% de EESA a ratas Zucker obesas (O) y sus controles delgadas (L), estableciéndose cuatro grupos de tratamiento (L1, L5, O1 y O5), además de sus grupos controles alimentados sólo con dieta estándar (LC y OC). Se controló de forma semanal el peso y la ingesta, así como la presión arterial sistólica mediante el sistema de pletismografía en la cola del animal consciente. Además se realizaron tests de tolerancia oral a la glucosa y resistencia a la insulina. Al finalizar el tratamiento, se midieron en suero parámetros como glucosa, insulina, CT, HDL-c, TG, NEFA, adiponectina y nitritos.

Como resultado se observó que, aunque no se vio afectada la evolución ponderal de los animales, el tratamiento con EESA atenuó significativamente y de forma concentración-

dependiente la hipertensión moderada que alcanzó el grupo control obeso. Los animales tratados con EESA 5% no desarrollaron resistencia a la insulina, síntoma que si presentaron tanto el grupo control obeso como el tratado con EESA 1%. En relación al análisis bioquímico, los niveles de CT, HDL-c, TG e insulinemia mejoraron de forma significativa y concentración-dependiente en los animales tratados. En el caso de EESA 5% se redujeron significativamente el índice aterogénico, HOMA-IR, así como TG y CT en hígado. Por último, la administración de EESA fue capaz de restaurar los niveles de adiponectina sérica en los animales obesos tratados, acercándose a los valores de los controles delgados. En el caso de los nitritos plasmáticos, se redujeron los niveles en los animales obesos tratados de forma significativa y concentración-dependiente.

En resumen, se puede concluir que el tratamiento crónico con una dieta suplementada con EESA mejora el perfil bioquímico y metabólico asociado al síndrome metabólico que se desarrolla en ratas Zucker obesas. Estas mejoras en el estado patológico se generan por una atenuación de las dislipemias, la insulinorresistencia y la hipertensión moderada que se da en el animal obeso. Además, este tratamiento con EESA es capaz de restaurar los niveles plasmáticos de biomarcadores en suero como son la adiponectina y los nitritos. En base a los efectos descritos se puede afirmar que, siendo necesario profundizar en los mecanismos de acción de EESA, se trata de un posible alimento funcional susceptible de ser utilizado en la prevención del síndrome metabólico y sus complicaciones asociadas.

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ORIGINAL CONTRIBUTION

Water-soluble rice bran enzymatic extract attenuates dyslipidemia, hypertension and insulin resistance in obese Zucker rats

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Abstract
Background and purpose Rice bran enzymatic extract (RBEE) has advantages compared to the original rice bran or its oils including water solubility, lack of rancidity and increased content in high nutritional proteins and nutraceutical compounds, particularly phytosterols, γ-oryzanol and tocols. Our aim was to determine the beneficial effects of RBEE in the pathogenesis of metabolic syndrome in obese Zucker rats.
Methods Obese Zucker rats and their lean littermates were fed a 1 and 5 % RBEE-supplemented diet (O1, O5, L1 and L5). Simultaneously, obese and lean Zucker rats, fed a standard diet, were used as controls (OC and LC, respectively). Body weight, food and water intake, and systolic blood pressure were weekly evaluated. After treatment, biochemical assays of serum glucose, insulin, triglycerides (TG), total cholesterol (TC), non-esterified fatty acids (NEFA), adiponectin and nitrates ($NO_{(x)}$) were determined.
Results RBEE treatment reduced circulating levels of TG and TC, whereas increased HDL-cholesterol without altering NEFA values in obese rats. The extract also induced a significant dose-dependent reduction of hypertension linked to obesity. RBEE of 5 % improved insulin resistance and subsequently reduced HOMA-IR index without altering serum glucose levels. Obese animals treated with RBEE showed partial restoration of adiponectin levels and a significant attenuation of pro-inflammatory values of $NO_{(x)}$.
Conclusion These findings evidence the nutraceutical properties of RBEE against the pathogenesis of metabolic syndrome by attenuating dyslipidemia, hypertension and insulin resistance as well as by restoring hypoadiponectinemia associated to obesity.

Keywords Rice bran · Metabolic syndrome · Dyslipidemia · Hypertension · Insulin resistance · Obese Zucker rats

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Introduction

Rice bran (RB) is a by-product of the rice milling, which derives from the outer layer of the rice grain. It is composed by the aleurone layer of the rice kernel and some part of the endosperm and germ. RB is an important source of fat, proteins and bioactive molecules with special interest as antioxidants and lipid-lowering compounds including γ-oryzanol (a mixture of ferulic acid esters of triterpene alcohols and sterols), tocols (tocopherols and tocotrienols) and unsaturated fatty acids [1, 2]. Chemical studies indicate that RB is specially rich in the phenolic compounds oryzanol and ferulic acid, which have demonstrated hypolipidemic effects—reducing total plasma cholesterol (TC) and triglyceride (TG) levels, and increasing high-density lipoprotein (HDL) levels—by mechanisms related to a strong antioxidant activity in rodents, rabbits, primates and humans [3]. In addition, proteins from RB are highly nutritional and have functional properties. Particularly, their characteristic activity as being hypoallergenic, hypocholesterolemic and anti-tumoral make them a superior

cereal protein that may find a wide range of applications (see review [4]).

Although RB shows an important composition in natural antioxidants and nutritional proteins [1, 4], its potential use as a functional food is limited by high insolubility of its protein as well as the integrity of its nutraceutical components, particularly referring to the phenolic fraction. Therefore, in the last years, oil derived from RB has been the most common form considered to study the therapeutic potential of RB. In fact, RB oil has demonstrated hypolipidemic [5–9] and hypoglycemic activities [10–13] in rodents and humans. Despite of these promising properties of RB oils, tendency to rancidity and the difficulty of administration have made necessary to obtain RB extracts with better physical and chemical properties.

Now, thanks to a novel enzymatic process it has been produced a water-soluble rice bran enzymatic extract (RBEE), which preserves functional properties and improves solubility of proteins and antioxidant components of RB [14, 15]. Besides, lipase inactivation during this enzymatic extraction avoids the problem of rancidity of RB [15]. The enzymatic treatment also increases concentrations of protein and minor functional components, especially γ-oryzanol and tocols, which are more than threefold higher compared to the original raw material RB [15, 16].

The preparation of products enriched with the antioxidant, hypolipidemic and hypoglycemic components of RB, which is the case for RBEE, may be of great interest for the treatment of chronic diseases showing oxidative stress, lipid and glucose/insulin disturbances as well as cardiovascular disorders. Metabolic syndrome has been described as a combination of several of these clinically specific risk features including obesity (central adiposity), dyslipidemia, insulin resistance, glucose intolerance, hypertension and non-alcoholic fatty liver disease and is becoming an important health problem worldwide [17, 18]. It has been established that dietary and physical activity are the first choice for improving or alleviating metabolic syndrome symptoms [19, 20]. Thus, the investigation of food components that may deal with the metabolic syndrome features is an important field to facilitate dietary-based therapies. Nowadays, dietary sources of natural antioxidants are of great interest due to the described association of the manifestations of metabolic syndrome and oxidative stress [20, 21].

Therefore, the potential antioxidant profile of RB and its components joined with their positive effects on lipid and glucose metabolism, led us to evaluate for the first time the effects of RB, in form of the water-soluble enzymatic extract RBEE, in the main clinical and biochemical manifestations of the metabolic syndrome developed in obese Zucker rats.

Materials and methods

Preparation and composition of RBEE

RBEE was prepared according to an enzymatic process previously described [15]. Briefly, RB was modified by enzymatic hydrolysis by using an endoprotease mixture as hydrolytic agent in a bioreactor with controlled temperature (60 °C) and pH (pH 8) and using the pH-stat method. The processing of this product follows different steps including centrifugation, filtration and concentration. The final product is brown syrup completely soluble in water. RBEE was chemically characterized by using AOAC standard protocols (Association of Official Analytical Chemists).

Chemical composition of RBEE has been previously characterized by Parrado et al. [15, 16]. Briefly, protein is the major component (38 %) in the form of peptides and free aminoacids due to the use of proteases for RB stabilization and with the aim of extract, solubilize and hydrolyze the initial insoluble proteins. The fat components present in RBEE (30 %) are mainly soluble because of protein interactions. Minor functional components of lipid fraction in RBEE include phytosterols (4,084 mg/kg), γ-oryzanol (1,260 mg/kg), tocopherols (99 mg/kg) and tocotrienols (174 mg/kg).

Animals and diets

Obese Zucker rats and their littermate controls, lean Zucker rats (8 weeks aged, Charles River Laboratories, Barcelona, Spain), were fed standard diet and water ad libitum. Obese and lean rats were divided into three groups and daily treated with either 1 % RBEE (O1 and L1) and 5 % RBEE supplementation (O5 and L5), or standard diet (SD) (OC and LC) ($n = 7$, each). Treatment with RBEE was administered during 20 weeks as a syrup form included in SD, supplemented with the concentrations indicated above. RBEE was extracted and supplied by the Enzymatic Production Technology group of the Department of Biochemistry and Molecular Biology, School of Pharmacy, University of Seville (Spain).

Body weight, food and water intake, and systolic blood pressure were weekly evaluated. Blood pressure and heart rate were measured by the pneumatic "*tail-cuff*" method with pressure meter (Niprem 645, Cibertec, Madrid, Spain). At the end of treatment, the animals were kept during 12 h fasting and were anesthetized with chloral hydrate 12 % intraperitoneally. Blood samples were collected by intracardiac puncture for biochemical assays in serum. The animals were then sacrificed, and visceral and epididymal adipose tissue (VAT and EAT, respectively), heart, pancreas, spleen, skeletal muscle and liver were

removed and weighted. The protocol for animal handling and experimentation agreed with the European Union European Community guidelines for the ethical treatment of animals (EEEC Directive of 1986; 86/609/EEC) and was approved by the Ethical Committee for Animal Research of the University of Seville.

Blood biochemical assays

Serum samples were obtained from blood by centrifugation for 20 min at 4,000 rpm and room temperature. Fasting glucose, total- and HDL-cholesterol were assessed by UV/visible spectrophotometry kits (Spin React, CIMA Diagnostics, Girona, Spain). TG and non-esterified fatty acids (NEFA) were also determined by commercial kits (WAKO Diagnostics, Richmond, VA, USA). Plasma adiponectin and insulin were measured by commercial enzyme-linked immunosorbent assay (ELISA) kits (B-Bridge International, Otsuka, Japan; Millipore, Missouri, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) and glucose tolerance was calculated as previously described [22]. Serum levels of nitric oxide metabolites ($NO_{(x)}$) were determined by using nitrate reductase to specifically reduce nitrate to nitrite; the latter was quantified by a colorimetric assay using the Griess reagent. Absorbance was measured spectrophotometrically at 550 nm [23].

Measurement of liver TG and TC

Extraction of lipids from livers was based on the method of Folch et al. [24]. Briefly, liver tissue (150 mg) was homogenized in 3 mL chloroform:methanol 2:1 (v/v) in a Polytron disrupter. The homogenate was centrifuged at 3,500 rpm for 10 min, and the supernatant fraction was collected. After several centrifugations with different solvents, the lipidemic fraction was obtained, and TG and TC contents in the liver were measured with the commercial kits named before.

Glucose and insulin resistance tests

At 20 weeks of treatment, rats were subjected to oral glucose tolerance and insulin resistance tests. The oral glucose tolerance test was performed by oral administration of glucose (2 g/kg body weight) to experimental groups previously fasted for 14 h. Blood samples were obtained from the tail vein before and after 30, 90 and 120 min of glucose administration. Plasma glucose concentration was determined using a blood glucose commercial monitoring meter (Accutrend® GCT; Roche Diagnostics, Barcelona, Spain). For insulin resistance test, food was withdrawn 3 h before the test and the rats were injected intraperitoneally with insulin (100 IU/mL; Humulina Regular®, Lilly S.A., Spain). Blood samples were collected at the same time intervals. The area under the glucose curve from both tests was calculated using Prism GraphPad 5.01 software (San Diego, CA, USA). For glucose tolerance data, each value is the total area under glucose curve for each animal and represents glucose changes from baseline during the test. For insulin resistance test, the area increment above the glucose curve was calculated, and each value represents the area relative to the time = 0 value for each animal.

Expression of results and statistical analysis

Data represented are mean ± SEM of $n = 7$ rats. One-way ANOVA with Tukey's comparison was used to compare data. Differences were considered significant when $P < 0.05$. A Prism GraphPad 5.01 software (San Diego, CA, USA) was used for statistical analysis.

Results

Body weight, food intake and organ weights

The body weight in obese Zucker rats was significantly higher ($P < 0.001$) than their lean littermates during the treatment (Table 1, Fig. 1). Treatment with RBEE did not modify body weights of both rat strains (Table 1, Fig. 1), excepting for L1, which final body weight attenuation was accompanied by a significant reduction in both food and caloric intake (Table 1). The average weekly food and caloric intake throughout the experimental period was also higher in obese Zucker rats than their matched lean littermates and was not altered by RBEE treatment (Table 1).

After treatment, liver and VAT relative weights (g/100 g body) resulted increased in OC versus LC ($P < 0.01$; $P < 0.001$), whereas skeletal muscle relative weight of OC was significantly reduced. RBEE treatment was able to significantly attenuate relative weights of the liver from obese animals compared to OC (Table 1). In addition, obese rats fed RBEE showed increased values of skeletal muscle relative weights, reaching similar values to those obtained in lean rats (Table 1). No changes were appreciated in heart, pancreas, spleen and EAT relative weights in both strains of animals (Table 1).

Systolic blood pressure changes

Obese Zucker rats showed moderately higher systolic blood pressure values than their lean littermates at the end of the treatment ($P < 0.001$). Administration of RBEE was able to reduce blood pressure values in O1 and O5, being

Table 1 Food and caloric intake, body weight and relative organ weights of the different groups of treatment at the end of the study. Mean ± SEM ($n = 7$)

	LC	L1	L5	OC	O1	O5
Food intake (g/wk/r)	154 ± 3[a]	127 ± 3[b]	142 ± 3[a]	175 ± 8[c]	162 ± 9[c]	161 ± 6[c]
Caloric intake (kcal)	452 ± 8[a]	375 ± 9[b]	445 ± 8[a]	508 ± 23[c]	478 ± 27[c]	506 ± 20[c]
Final body weight (g)	461 ± 11[a]	432 ± 7[b]	490 ± 11[a]	569 ± 21[c]	606 ± 27[c]	601 ± 29[c]
Organ weight (g/100 g body weight)						
Heart (g)	0.30 ± 0.01[a]	0.29 ± 0.01[a]	0.27 ± 0.01[a]	0.28 ± 0.01[a]	0.26 ± 0.01[a]	0.24 ± 0.01[a]
Pancreas (g)	0.16 ± 0.02[a]	0.22 ± 0.02[a]	0.25 ± 0.01[a]	0.13 ± 0.01[a]	0.12 ± 0.02[a]	0.16 ± 0.01[a]
Spleen (g)	0.150 ± 0.001[a]	0.130 ± 0.001[a]	0.140 ± 0.001[a]	0.17 ± 0.01[a]	0.16 ± 0.01[a]	0.150 ± 0.001[a]
Skeletal muscle (g)	1.28 ± 0.09[a]	1.66 ± 0.11[b]	1.92 ± 0.13[b]	0.79 ± 0.04[c]	1.07 ± 0.04[d]	1.17 ± 0.06[d]
Liver (g)	2.96 ± 0.14[a]	2.8 ± 0.2[a]	2.8 ± 0.2[a]	5.9 ± 0.3[b]	4.6 ± 0.2[c]	4.6 ± 0.3[c]
VAT (g)	1.61 ± 0.13[a]	1.36 ± 0.10[a]	1.50 ± 0.07[a]	2.7 ± 0.3[b]	3.10 ± 0.13[b]	3.2 ± 0.2[b]
EAT (g)	1.07 ± 0.10[a]	0.84 ± 0.07[a]	0.97 ± 0.06[a]	0.96 ± 0.06[a]	1.26 ± 0.09[a]	1.40 ± 0.10[a]

Values in a row without a common superscript letter differ significantly ($P < 0.05$)

LC lean controls fed standard diet (SD), *L1* lean fed 1 % RBEE-supplemented diet, *L5* lean fed 5 % RBEE-supplemented diet, *OC* obese controls fed SD, *O1* obese fed 1 % RBEE-supplemented diet, *O5* obese fed 5 % RBEE-supplemented diet

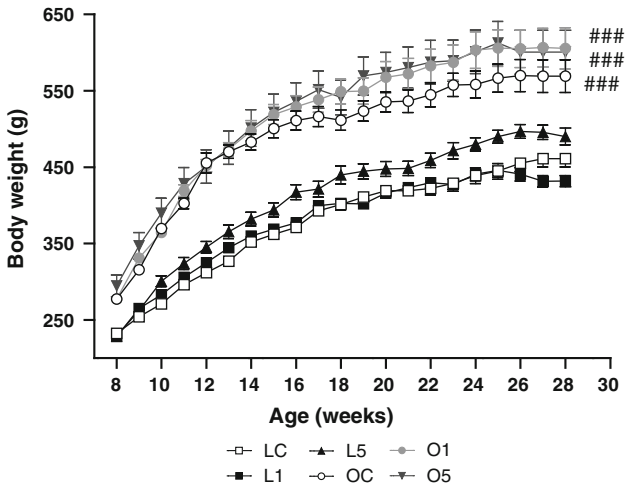

Fig. 1 Body weight evolution of Zucker rats during 20 weeks of treatment of receiving either standard diet (SD) or rice bran enzymatic extract (RBEE)-supplemented diet. Experimental groups: *LC* lean controls fed SD, *L1* lean fed 1 % RBEE-supplemented diet, *L5* lean fed 5 % RBEE-supplemented diet, *OC* obese controls fed SD, *O1* obese fed 1 % RBEE-supplemented diet, *O5* obese fed 5 % RBEE-supplemented diet. Data are mean ± SEM ($n = 7$). $^{###}P < 0.001$ versus LC

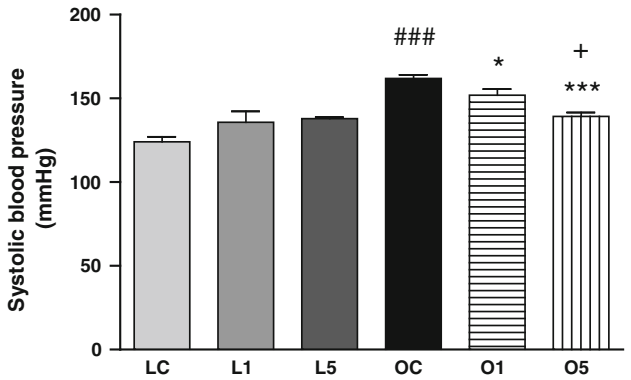

Fig. 2 Final values of systolic blood pressure after 20 weeks of treatment with either standard diet (SD) or rice bran enzymatic extract (RBEE)-supplemented diet. *LC* lean controls fed SD, *L1* lean fed 1 % RBEE-supplemented diet, *L5* lean fed 5 % RBEE-supplemented diet, *OC* obese controls fed SD, *O1* obese fed 1 % RBEE-supplemented diet, *O5* obese fed 5 % RBEE-supplemented diet. Data are mean ± SEM ($n = 7$). $^{###}P < 0.001$ versus LC; $^{*}P < 0.05$, $^{***}P < 0.001$ versus OC; $^{+}P<0.05$ versus O1

significantly most effective the high concentration ($P < 0.001$) (Fig. 2).

Lipid profile

Serum analysis showed higher concentrations of TC, HDL, TG and NEFA in obese compared with lean groups ($P < 0.001$) (Table 2). RBEE-supplemented diet produced a significant reduction of parameters such as TC, TC/HDL-C ratio and TG values, being O5 the group with the most important improvement in the lipid profile ($P < 0.001$). RBEE induced no changes in NEFA values in serum.

As shown on Table 2, ALT values were higher in obese Zucker rats than in the lean strain ($P < 0.001$ vs LC), whereas no differences were appreciated between both strains of animals regarding to AST serum levels. Neither ALT nor AST serum levels were modified by the RBEE treatment.

The hepatic lipid fraction obtained from obese rats was significantly higher than that from their lean littermates ($P < 0.001$ vs LC). Hepatic level of TG was greater in OC than LC ($P < 0.05$), whereas this value was notably attenuated by administration of 5 % RBEE in obese rats ($P < 0.01$ vs OC) (Table 2). In contrast, cholesterol levels

Table 2 Biochemical analysis after chronic treatment with RBEE. Mean ± SEM ($n = 7$)

	LC	L1	L5	OC	O1	O5
Serum						
TC (mmol/L)	2.35 ± 0.10^a	2.4 ± 0.2^a	2.59 ± 0.06^a	6.0 ± 0.3^b	5.4 ± 0.2^c	5.0 ± 0.7^d
HDL-C (mmol/L)	0.88 ± 0.07^a	0.95 ± 0.11^a	0.91 ± 0.13^a	2.4 ± 0.3^b	2.9 ± 0.2^c	3.2 ± 0.3^d
TC/HDL-C	2.7 ± 0.2^a	2.7 ± 0.2^a	3.0 ± 0.3^a	3.2 ± 0.8^b	2.03 ± 0.13^c	1.7 ± 0.2^c
TG (mmol/L)	0.98 ± 0.15^a	0.66 ± 0.08^a	0.66 ± 0.07^a	4.6 ± 0.6^b	4.0 ± 0.2^c	3.30 ± 0.15^d
NEFA (mmol/L)	0.49 ± 0.04^a	0.45 ± 0.14^a	0.47 ± 0.04^a	0.94 ± 0.10^b	0.91 ± 0.03^b	0.99 ± 0.11^b
Glucose (mmol/L)	6.6 ± 0.6^a	5.2 ± 0.6^b	5.7 ± 0.3^a	8.7 ± 1.0^c	9.4 ± 0.5^c	9.0 ± 1.0^c
Insulin (ng/mL)	0.48 ± 0.12^a	0.42 ± 0.11^a	0.48 ± 0.08^a	4.5 ± 0.6^b	4.1 ± 0.5^c	2.6 ± 0.6^d
HOMA-IR	2.6 ± 0.5^a	3.8 ± 0.9^a	3.0 ± 0.4^a	46 ± 7^b	37 ± 4^b	22 ± 6^c
ALT	74.8 ± 0.5^a	73.1 ± 1.0^a	67.3 ± 0.8^a	90 ± 4^b	87 ± 2^b	93 ± 6^b
AST	141 ± 6^a	136 ± 3^a	131 ± 2^a	1 26 ± 9^a	1 24 ± 6^a	1 20 ± 8^a
Liver						
Total lipids (mg)	76 ± 2^a	81 ± 3^a	92.6 ± 0.9^b	102 ± 4^c	99 ± 3^c	102 ± 3^c
TG (mg/g)	24.8 ± 1.4^a	23 ± 3^a	38 ± 2^b	31.9 ± 0.8^c	35 ± 2^c	19.17 ± 0.04^d
TC (mg/g)	11.0 ± 0.2^a	29 ± 2^b	30 ± 3^b	9.6 ± 0.8^a	9 ± 2^a	20 ± 2^c

Values in a row without a common superscript letter differ significantly ($P < 0.05$)

LC lean controls fed SD, *L1* lean fed 1 % RBEE-supplemented diet, *L5* lean fed 5 % RBEE-supplemented diet, *OC* obese controls fed SD, *O1* obese fed 1 % RBEE-supplemented diet, *O5* obese fed 5 % RBEE-supplemented diet. *TC* total cholesterol, *HDL-C* high-density lipoprotein-cholesterol, *TG* triglycerides, *NEFA* non-esterified fatty acids, *HOMA-IR* homeostatic model assessment-insulin resistance

in liver resulted increased in both lean and obese rats treated with 5 % RBEE (Table 2).

Glucose and insulin tests

As shown in Table 2, RBEE of 5 % administration induced an attenuation in HOMA-IR values of obese rats ($P < 0.01$). This fact was according to a reduction in serum insulin levels in O5 ($P < 0.001$ vs OC and O1) while fasting glucose levels in obese groups remained unaltered. Plasma glucose concentrations that obtained in the oral glucose tolerance test at 20 weeks of treatment were slightly different between lean and obese rats (Fig. 3a). In addition, the analysis of the area under the plasma glucose curve confirmed that OC presented a deteriorated glucose tolerance compared with LC (Fig. 3c). Treatment with RBEE tended to improve glucose tolerance, but the difference versus obese control was not significant (Fig. 3c).

Insulin resistance test evidenced the development of this symptom in OC, which presented an increment of the area under the plasma glucose curve significantly lower than LC at 20 weeks of treatment ($P < 0.05$) (Fig. 3b, d). RBEE of 5 % led to a significant recovering of insulin sensibility in obese rats ($P < 0.001$ vs OC) (Fig. 3b, d).

Inflammatory factors related to obesity

Serum adiponectin values were notably reduced in OC compared to LC group ($P < 0.01$) (Fig. 4a). However, this biomarker of obesity was significantly recovered in/by treatment with RBEE ($P < 0.05$).

Serum levels of $NO_{(x)}$ were fivefold increased in obese than lean rats ($P < 0.001$) (Fig. 4b). Both concentrations of RBEE induced a significant attenuation in this parameter in obese rats ($P < 0.001$), being 5 % RBEE supplementation significantly more effective than 1 % ($P < 0.001$).

Discussion

Metabolic syndrome is a controversial clinical entity characterized by a number of cardiometabolic risk factors that include obesity, dyslipidemia, insulin resistance and hypertension. This clustering of risk factors is related to an increased risk of cardiovascular diseases and diabetes [17, 25]. Although insulin and other pharmacological strategies are able to control many aspects of diabetes, they inadequately prevent cardiovascular complications associated to metabolic syndrome [25]. Otherwise, the first strategy in the prevention of these cardiometabolic disorders consists of including in the diet food or dietary components with functional properties [19–21].

Rice, and particularly RB, is an excellent nutritional source of bioactive compounds, including high healthy value proteins and phytochemicals such as γ-oryzanol, sterols and tocols [2, 4]. Nevertheless, the use of RB as a functional food is limited by insolubility of its major components, rapid development of rancidity and possible

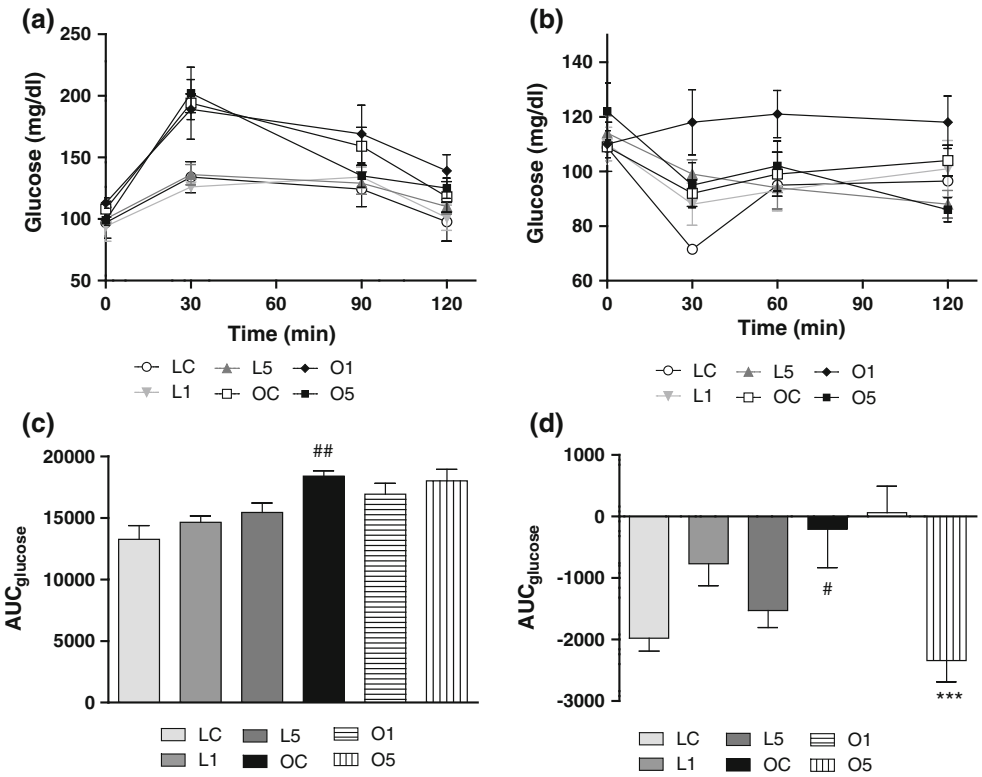

Fig. 3 Profile of serum glucose changes obtained from oral glucose tolerance (**a**) and insulin resistance tests (**b**) at 20 weeks of treatment with either standard diet (SD) or RBEE-supplemented diet. Area under curve (AUC) that results of serum glucose concentrations in the glucose tolerance test (**c**) and AUC increment obtained from insulin resistance test (**d**). *LC* lean controls fed SD, *L1* lean fed 1 % RBEE-supplemented diet, *L5* lean fed 5 % RBEE-supplemented diet, *OC* obese controls fed SD, *O1* obese fed 1 % RBEE-supplemented diet, *O5* obese fed 5 % RBEE-supplemented diet. Data are mean ± SEM ($n = 7$). $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ versus LC; $^{***}P < 0.001$ versus OC

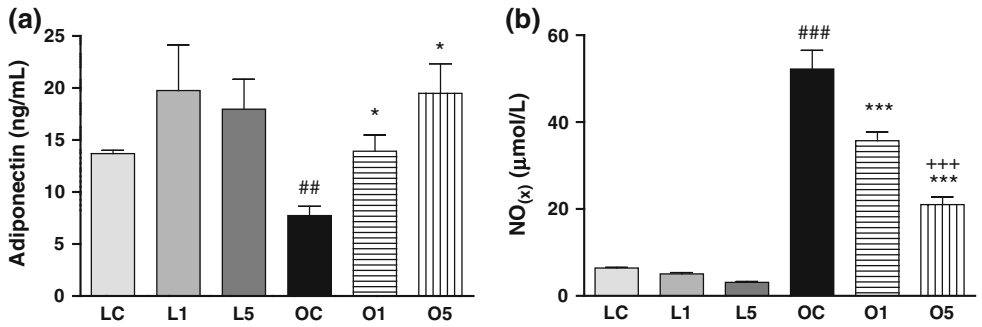

Fig. 4 Serum adiponectin (**a**) and nitrates/nitrites ($NO_{(x)}$) (**b**) values after 20 weeks of treatment with either standard diet (SD) or rice bran enzymatic extract (RBEE)-supplemented diet in lean and obese Zucker rats. *LC* lean controls fed SD, *L1* lean fed 1 % RBEE-supplemented diet, *L5* lean fed 5 % RBEE-supplemented diet, *OC* obese controls fed SD, *O1* obese fed 1 % RBEE-supplemented diet, *O5* obese fed 5 % RBEE-supplemented diet. Data are mean ± SEM ($n = 7$). $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus LC; $^{*}P < 0.05$, $^{***}P < 0.001$ versus OC; $^{+++}P < 0.001$ versus O1

hull contamination [26, 27]. These limitations have been counteracted by recent production of an RB extract by enzymatic hydrolysis, so called RBEE, which has important advantages over raw material or oils regarding water solubility, increased content in nutraceutical compounds and lack of rancidity [15, 16]. Recently, RBEE has demonstrated antioxidant and hypocholesterolemic activities in vivo [15, 16] that have been mainly attributed to the presence of γ-oryzanol. Also, phytosterols content has been associated to lipid-lowering action of RBEE and the presence of tocols and sulfur aminoacids to the antioxidant activity [15, 16].

In our study, we demonstrate for the first time how long-term administration of a diet supplemented in RBEE improves biochemical and metabolic disturbances associated with metabolic syndrome in obese Zucker rats by attenuating dyslipidemia, insulin resistance and hypertension as well as restoring plasmatic levels of adiponectin and $NO_{(x)}$.

In obese Zucker rats, increased adipose tissue and peripheral insulin resistance are associated with an augmented liver lipogenesis and elevated NEFA levels in serum, thus leading to ectopic depot of TG in the liver with increased circulating levels of TG and TC [28].

As expected, RBEE treatment reduced hypertriglyceridemia and hypercholesterolemia in obese Zucker rats, in a dose-dependent manner, and without altering serum NEFA values. These responses were accompanied by a significant increase of HDL-cholesterol levels. The decrease in serum and liver TG induced by RBEE, particularly at 5 %, could be related to the reduction in liver weight observed in this experimental group, since this fact cannot be a consequence of a reduction in body weight. In addition to the liver weight reduction, no evidence of hepatic steatosis was observed since levels of transaminases remained unaltered by RBEE.

The lipid-lowering property of RBEE treatment in Zucker rats agrees with previous studies using the same enzymatic extract in rats receiving high cholesterol-rich diets [16], as well as other investigations with RB oils in hypercholesterolemic rodents and humans [5–9]. The main responsible for this beneficial effect of RBEE and other RB extracts on lipid profile may be its phytosterol content. The major sterol on RBEE is 4-desmethyl sterols, β-sitosterol, which is very effective in competing with cholesterol for incorporating in mixed micelles [29]. The hypolipidemic action of the enzymatic extract may also be related to its important content of γ-oryzanol. Several mechanisms have been suggested to be involved in this beneficial effect of γ-oryzanol including a decrease in cholesterol absorption and an increased bile flow or rise in cholesterol fecal excretion [3]. Furthermore, γ-oryzanol may reduce levels of cholesterol by attenuating apolipoprotein B synthesis in humans [30]. It has also been described that triterpene alcohols from γ-oryzanol evoke hypocholesterolemic activity by themselves [31].

Moreover, this lipid-lowering action might be also related to the potential hypocholesterolemic and anti-atherogenic capacity of proteins from RB [4, 32], since solubility and bioactivity of these proteins is notably enhanced in RBEE. The beneficial actions of RB proteins against hyperlipemia and atherogenesis have also been related to the high concentration of L-arginine in the protein fraction of RBEE, which is a very effective aminoacid in this regard [15, 16].

Tocotrienols from RB, in addition to its antioxidant property, do also lower serum cholesterol by an inhibition of the regulatory enzyme hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase thus decreasing hepatic cholesterol synthesis [33, 34]. In contrast to this action, α-tocopherol has an inductor effect on this enzyme activity [34]. Since tocotrienols are also converted to tocopherols in vivo, it is necessary not to exceed a certain dose, as this could be a counter-productive. This fact could be related to the effect of the higher dose tested of RBEE on hepatic cholesterol levels. Zucker obese rats fed 5 % RBEE showed augmented levels of liver cholesterol, despite of the plasmatic cholesterol decreasing. This dose of RBEE may be excessive in terms of tocotrienols that might be converted into tocopherols thus inducing the activity of HMG-CoA reductase with the consequent increase in liver cholesterol. Although the concentrations of RBEE used in this study seem to be suitable to improve lipid profile in obese Zucker rats, the higher dose of 5 % RBEE could produce certain cholesterol accumulation in the liver of this animal model.

Obesity is a well-established risk factor for the development of hypertension, since there is a strong correlation between overweight and hypertension [35]. In fact, the increase in blood pressure developed by fatty animals was higher than that found in their littermates lean rats. Obesity-related hypertension is associated with both a decrease in adiponectin concentration and the development of insulin resistance, which are in turn related [36]. The reasons for the association of insulin resistance and essential hypertension can be sought in at least four general types of mechanisms: Na^+ retention, sympathetic nervous system overactivity, disturbed membrane ion transport and proliferation of vascular smooth muscle cells [36]. Also, hypertension related to obesity has been related to vascular and endothelial dysfunction due to decreased endothelial NO release by down-regulation of endothelial NO synthase as well as increased oxidative stress leading to an augmented breakdown of endothelial NO, independently of NO metabolites produced by inducible NOS (iNOS) in fat or muscle [37]. Additional mechanisms such as increased generation of angiotensin II and endothelin-1 seem to contribute to arterial hypertension in obese Zucker rats [37]. According to our results, RBEE had a concentration-dependent positive effect on systolic blood pressure, insulin resistance and adiponectin levels. That is, RBEE-supplemented diet of 5 % induced a stronger reduction in systolic blood pressure of obese treated-rats than RBEE of 1 %. At the same way, in the insulin resistance test, serum glucose was recovered to normal levels in O5, as well as in the case of serum adiponectin values. These findings may be related to the high content of RBEE in γ-oryzanol, since it has been recently reported that this compound is able to recover mice-induced hypoadiponectinemia thus improving insulin sensibility [38]. On the other hand, there are several studies that confirm the capacity of RB to reduce blood pressure in genetic models of hypertensive rats, being ferulic acid the main responsible for this action [39, 40]. Besides, single administration of ferulic acid to spontaneously hypertensive rats induced an attenuation of blood pressure by inhibiting plasmatic activity of angiotensin-1-converting enzyme activity [41].

We also found that RBEE ameliorated insulin resistance in obese Zucker rats. Although the extract did not significantly change fasting glucose, insulin resistance test data

strongly indicate the beneficial effect of RBEE on this regard. Qureshi and coworkers demonstrated that fractions from stabilized RB extract rich in tocopherols/tocotrienols can effectively control blood glucose levels and insulin resistance in diabetic humans [10, 34]. They propose a synergistic effect of several RB bioactive components, particularly tocols and γ-oryzanol, which has potent antioxidant activity thus ameliorating complications of diabetes such as glycation, glycoxidation and hyperlipemia as well as exerting indirect effects on glucose absorption, utilization or excretion [10, 12, 34]. Also, proteins derived from the enzymatic treatment of RB might be involved in the beneficial effect of RBEE in glucose and insulin metabolism, since these proteins may attenuate formation of advanced glycosylation end products, which are increased under a diabetic state [10].

On the other hand, the attenuation induced by RBEE on insulin resistance of obese rats may be partially related to an increased level of adiponectin in treated obese animals. Obese animals and humans are featured by a dysregulated production of hormones and adipocytokines secreted by adipose tissue, namely decreased adiponectin and overproduction of pro-inflammatory mediators such as iNOS and tumor necrosis factor (TNF)-α, leading to obesity-related insulin resistance and inflammation [42]. In our study, RBEE restored plasmatic adiponectin levels in obese Zucker rats, thus providing protection against the pathogenesis of metabolic syndrome. Regarding to the pro-inflammatory state associated with obesity in addition to the hypoadiponectinemia, in the present investigation, plasmatic values of $NO_{(x)}$ have been evaluated as a biomarker of iNOS induction in obese rats. RBEE diet improved the imbalanced production of $NO_{(x)}$ in obese animals, thus indicating the potential role of this enzymatic extract on systemic inflammation, which is a key mechanism in the pathogenesis of metabolic syndrome features [17]. The main constituent of RBEE involved in the potential action of the enzymatic extract on hypoadiponectinemia and inflammation related to obesity may be the content on γ-oryzanol, since recent studies has evidenced the beneficial effect of this component restoring adiponectin levels in obese mice [38] as well as its steryl ferulates on inflammatory markers involved in metabolic syndrome [43].

In summary, this investigation demonstrates that chronic administration of a novel water-soluble enzymatic extract of rice bran could be a suitable treatment for improving or alleviating metabolic syndrome-associated risk factors. This RBEE has shown physical and chemical advantages over raw material or RB oils regarding water solubility, lack of rancidity, and increased content in nutraceutical compounds such as γ-oryzanol, sterols and tocotrienols, thus leading to an important bioactivity in vivo. In this study, an RBEE-supplemented diet had positive effects in obesity-derived hyperlipidemia, hypertension and hyperinsulinemia. In addition, obese animals treated with RBEE showed a significant restoration of adiponectin levels and an attenuation of pro-inflammatory values of $NO_{(x)}$ that seem to be associated with obesity. All of these activities could be mainly attributed to the presence of γ-oryzanol and ferulic acid in RBEE and/or a synergistic effect of its nutraceutical compounds. Also, solubility of high nutritional proteins in the enzymatic extract could be involved in the beneficial effects.

Further studies are in progress to better clarify the mechanisms implicated in these beneficial actions and thus to reinforce the potential of RBEE as a functional food, with special emphasis in the pathogenesis of the metabolic syndrome and associated complications.

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ARTÍCULO 2: **Rice bran enzymatic extract restores endothelial function and vascular contractility in obese rats by reducing vascular inflammation and oxidative stress**

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Un extracto enzimático de salvado de arroz restaura la función endotelial y la contractilidad vascular en ratas obesas por una reducción de la inflamación vascular y el estrés oxidativo.

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En el estudio anterior, se observó cómo una dieta suplementada con un extracto enzimático de salvado de arroz (EESA), y administrada en un tratamiento prolongado, presentaba beneficios frente a las dislipemias, la hiperinsulinemia y la hipertensión arterial moderada asociada a la obesidad [116]. Otra de las consecuencias de la obesidad es el deterioro de la función vascular, y en este caso pensamos que podía ser interesante abordar una de las mayores manifestaciones patológicas del síndrome metabólico: la disfunción endotelial [83]. El endotelio ha sido ampliamente estudiado como un reflejo de problemas cardiovasculares, ya que en él se desarrollan procesos de gran importancia donde el óxido nítrico, especies reactivas de oxígeno o el factor de necrosis tumoral-alfa están involucrados. Fue por esto que el objetivo de este estudio fue investigar el efecto que el tratamiento con EESA tendría frente al daño vascular desarrollado en ratas Zucker obesas y evaluar el mecanismo por el cual el extracto ejerce su acción protectora.

Para ello, durante 20 semanas se les administró una dieta estándar enriquecida al 1% y 5% de EESA a ratas Zucker obesas (O), estableciéndose dos grupos de tratamiento (O1% y O5%), y otros dos grupos controles de ratas obesas (OC) y delgadas (LC) alimentadas sólo con dieta estándar. Al finalizar el tratamiento, se evaluó la función vascular mediante el montaje de anillos aórticos en baño de órganos. Se investigó la relajación dependiente e independiente de endotelio mediante la realización de curvas acumulativas de acetilcolina y nitroprusiato sódico, respectivamente. A su vez, se estudió el papel del óxido nítrico mediante inhibidores de la enzima óxido nítrico sintasa endotelial e inducible mediante la adición al baño de L-NAME y 1400W, respectivamente. En paralelo, se realizaron estos mismos ensayos enfocados a la contracción vascular. También se determinó la expresión vascular de eNOS mediante Western Blot, de iNOS por inmunofluorescencia, y de iNOS, TNF-α y las subunidades

de la NADPH oxidasa (NOX-1 y $p22^{phox}$) mediante PCR a tiempo real. Además, se determinó la producción vascular de aniones superóxido mediante la detección fluorescente de dihidroetidio.

Como resultado, se atenuó de forma significativa tanto la disfunción endotelial como la hiperreactividad vascular desarrollada por los animales obesos tratados con EESA 1%. En este aspecto pudo contribuir el aumento en la expresión proteica de eNOS en las arterias de los animales obesos tratados con el extracto, al igual que se restauraron los niveles de expresión vascular de iNOS y TNF-α. Además, el incremento del estrés oxidativo vascular que se observó en las arterias de los animales obesos controles fue disminuido de forma significativa gracias al tratamiento, hecho confirmado por la disminución significativa de la expresión génica de subunidades de la NADPH oxidasa como la NOX-1 y la $p22^{phox}$.

En conclusión, se puede decir que la administración en la dieta de un suplemento de EESA es capaz de restaurar la disfunción vascular asociada a la obesidad, además de reducir la expresión de biomarcadores pro-oxidantes y pro-inflamatorios en las arterias de conductancia de animales obesos. Estos datos, unidos a los obtenidos en los estudios previos donde se comprobaron las mejoras en el perfil lipídico, en la resistencia a la insulina y en los niveles de adiponectina y nitritos en suero, nos demuestran que EESA puede ser un candidato ejemplar para ser utilizado como alimento funcional en el tratamiento de las alteraciones vasculares que están ligadas al síndrome metabólico.

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Rice bran enzymatic extract restores endothelial function and vascular contractility in obese rats by reducing vascular inflammation and oxidative stress☆

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Abstract

Background: Rice bran enzymatic extract (RBEE) used in this study has shown beneficial activities against dyslipidemia, hyperinsulinemia and hypertension. Our aim was to investigate the effects of a diet supplemented with RBEE in vascular impairment developed in obese Zucker rats and to evaluate the main mechanisms mediating this action.

Methods and results: Obese Zucker rats were fed a 1% and 5% RBEE-supplemented diet (O1% and O5%). Obese and their lean littermates fed a standard diet were used as controls (OC and LC, respectively). Vascular function was evaluated in aortic rings in organ baths. The role of nitric oxide (NO) was investigated by using NO synthase (NOS) inhibitors. Aortic expression of endothelial NOS (eNOS), inducible NOS (iNOS), tumor necrosis factor (TNF)-α and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits and superoxide production in arterial wall were determined. Endothelial dysfunction and vascular hyperreactivity to phenylephrine in obese rats were ameliorated by RBEE treatment, particularly with 1% RBEE. Up-regulation of eNOS protein expression in RBEE-treated aortas should contribute to this activity. RBEE attenuated vascular inflammation by reducing aortic iNOS and TNF-α expression. Aortas from RBEE-treated groups showed a significant decrease of superoxide production and down-regulation of NADPH oxidase subunits.

Conclusion: RBEE treatment restored endothelial function and vascular contractility in obese Zucker rats through a reduction of vascular inflammation and oxidative stress. These results show the nutraceutical potential of RBEE to prevent obesity-related vascular complications.

Keywords: Obesity; Rice bran; Vascular dysfunction; Vascular inflammation; Vascular oxidative stress

1. Introduction

Occidental dietary habits have contributed to increased prevalence of obesity, type 2 diabetes mellitus and other pathologies included in the metabolic syndrome [1]. Obesity, in particular abdominal obesity, has been established as a primary contributor to acquired insulin resistance, as increasing adiposity is correlated with impaired insulin action. Endothelial dysfunction, an independent predictor of cardiovascular events [2], has been consistently associated with obesity and the metabolic syndrome [3] in a complex interplay with insulin resistance [4]. Nevertheless, it has been reported that vascular dysfunction of obesity is not only limited to the endothelium but also involves the smooth muscle cell layer, leading to an increased oxidative stress in the vascular wall and the subsequent deregulation of the main control mechanisms providing vascular homeostasis [5]. Vascular function impairment in the metabolic syndrome mainly implies an unbalance between the vasoprotective effect of endothelial nitric oxide (NO) and the unfavorable action of vasoconstrictor factors [e.g., endothelin-1 and reactive oxygen species (ROS)] and proinflammatory mediators [e.g. tumor necrosis factor (TNF)-α] [5]. The pathophysiology of obesity-related vascular dysfunction is therefore an important target for developing new therapeutic approaches aimed to ameliorate cardiovascular risk factors related to metabolic syndrome.

Numerous studies suggest that the first strategy in the prevention of disorders associated with obesity consists of including in the diet food or dietary components with functional properties [6,7]. Rice, and particularly rice bran, is an excellent nutritional source of bioactive compounds, including high-healthy-value proteins and phytochemicals such as γ-oryzanol, sterols and tocols [8,9]. Besides, phenolic compounds contained in rice bran, such as γ-oryzanol and ferulic acid, are known to provide strong antioxidant activities [10,11]. The

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hypolipidemic, antioxidant and anti-inflammatory properties of the mixture of phytosteryl ferulates contained in γ-oryzanol make it a good candidate for health food [12,13]. However, the therapeutic use of rice bran is limited because of the insolubility of its proteins and the integrity of its nutraceutical compounds. For this reason, rice bran oils have become commonly utilized in order to study its properties, despite the high risk of rancidity that it involves [14]. These limitations have been counteracted by the recent production of a water-soluble rice bran enzymatic extract (RBEE) [15] that provides numerous advantages over other rice bran derivatives regarding water solubility, increased content in nutraceutical compounds and lack of rancidity.

Our recent investigations have evidenced that a diet supplemented with RBEE is able to ameliorate cardiometabolic risk factors in obese Zucker rats, showing a remarkable action on dyslipidemia, moderate hypertension, insulin resistance and adiponectin levels [16]. However, the effect of RBEE on vascular dysfunction associated with obesity and the main mechanisms by which this rice bran derivative induces its beneficial action on cardiometabolic risk factors remain unknown. The potential of rice bran extracts on vascular alterations has only been recently suggested in a few *in vitro* investigations which evaluated the effects of γ-oryzanol and a rice bran ethyl acetate extract on adhesion molecules expression in vascular endothelial cells or hypertrophy in smooth muscle cells, respectively [17,18].

Considering the beneficial effect of an RBEE-enriched diet on cardiometabolic parameters developed in obese rats, it is expected that RBEE will be able to restore endothelial dysfunction and vascular inflammation and oxidative stress associated with obesity in an animal model of metabolic syndrome. Thus, the aim of our study was to determine the capacity of a diet supplemented with RBEE to modify vascular dysfunction in obese Zucker rats and to identify the main pathways that could be implicated in the RBEE bioactivity. Elucidation of these vascular mechanisms might partially explain the beneficial action of RBEE treatment in cardiometabolic parameters of obese animals found in our previous investigations.

2. Materials and methods

2.1. Preparation and composition of RBEE

RBEE was prepared according to an enzymatic process previously described [15]. Briefly, RB was modified by enzymatic hydrolysis by using an endoproteases mixture as hydrolytic agent in a bioreactor with controlled temperature (60°C) and pH (pH 8) and using the pH-stat method. The processing of this product follows different steps including centrifugation, filtration and concentration. The final product is brown syrup completely soluble in water. RBEE was chemically characterized by using Association of Official Analytical Chemists standard protocols.

The chemical composition of RBEE has been previously characterized by Parrado et al. [15]. Briefly, protein is the major component (38%), in the form of peptides and free amino acids, due to the use of proteases for RB stabilization and with the aim of extracting, solubilizing and hydrolyzing the initial insoluble proteins. The fat components present in RBEE (30%) are mainly soluble because of protein interactions. Minor functional components of lipid fraction in RBEE include phytosterols (4084 mg/kg), γ-oryzanol (1260 mg/kg), tocopherols (99 mg/kg) and tocotrienols (174 mg/kg).

2.2. Animals and diets

Obese Zucker rats and their littermate controls, lean Zucker rats (8 weeks old; Charles River Laboratories, Barcelona, Spain) were fed standard diet and water *ad libitum*. Obese rats were divided into three groups (n=7) and treated daily with 1% RBEE supplementation (O1%), 5% RBEE supplementation (O5%) or standard diet (obese control group, OC). A group of lean Zucker rats (n=7) was also fed a standard diet (lean control group, LC). RBEE treatment was administered for 20 weeks in syrup form included in the standard diet supplemented with the concentrations indicated above. RBEE was extracted and supplied by the Enzymatic Production Technology group of the Department of Biochemistry and Molecular Biology (University of Seville, Spain). The selected doses of RBEE have shown beneficial effects in our previous study [16].

Body weight, food and water intake, and systolic blood pressure were evaluated weekly. At the end of treatment, animals were kept during 12 h fasting and were anesthetized with chloral hydrate 12% intraperitoneally. The protocol for animal handling and experimentation agreed with the European Union European Community guidelines for the ethical treatment of animals (EEEC Directive of 1986; 86/609/EEC) and was approved by the Ethical Committee for Animal Research of the University of Seville.

2.3. Tissue preparation

Thoracic aortas were dissected, cleaned and placed in cold modified Krebs–Henseleit solution (KHS) (in mmol/L: NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 1.8, KH_2PO_4 1.18 and glucose 11). Aortic rings to be used for detection of superoxide anion ($O_2^{\bullet-}$) were maintained in KHS containing 30% sucrose overnight, placed into cryomolds containing Tissue-Tek OCT embedding medium (Sakura Finetek Europe, the Netherlands) and immediately frozen in liquid nitrogen for storage at −80°C [19]. For immunofluorescence studies, aortas were fixed in 4 % phosphate-buffered paraformaldehyde (pH 7.4) for 1 h and then washed in phosphate-buffered saline (PBS). Afterwards, arterial segments were placed in 30% sucrose PBS overnight, transferred to cryomolds with Tissue Tek OCT embedding medium, frozen in liquid nitrogen and kept at −80°C until analysis [20].

2.4. Reactivity experiments

Aortic rings were disposed in organ baths, and mechanical activity was measured as previously described [19]. Contractile capacity of the vessels was assessed with either KCl 80 mmol/L or phenylephrine (Phe) 0.3 μmol/L prior to contraction or relaxation experiments, respectively. The presence of functional endothelium was evaluated by the ability of acetylcholine (ACh) 1 μmol/L to induce more than 50% relaxation of precontracted vessels (8 of 130 aortic rings were excluded).

For the experiments, aortic segments were exposed to cumulative concentrations of Phe (0.001–10 μmol/L) to obtain concentration–response curves. Endothelium-dependent and -independent vasodilatations were studied by evaluating the relaxation induced by ACh (0.001–10 μmol/L) in endothelium-intact [E(+)] rings and sodium nitroprusside (SNP, 0.1–100 nmol/L) in endothelium-denuded [E(−)] rings in Phe-precontracted arteries. Concentration–response curves were constructed in the absence or presence of the nonselective NO synthase (NOS) inhibitor *N*-nitro-L-arginine methyl ester (L-NAME, 300 μmol/L) and the selective inducible NOS (iNOS) inhibitor 1400W (30 μmol/L).

2.5. Western blot analysis

Protein fraction was purified from aortas after guanidine hydrochloride extraction of RNA and DNA. After isolation by ethanol precipitation, proteins were dissolved in sodium dodecyl sulfate (SDS) 4%–urea 1 M. Protein (40 μg) was resolved by electrophoresis on SDS polyacrylamide gel electrophoresis gels and transferred onto nitrocellulose membranes. Immunoblotting was performed using a specific primary endothelial NOS (eNOS) antibody (1:800 dilution; Cell Signaling Technology, Beverly, MA, USA) at 4°C overnight. Bands were visualized by enhanced chemiluminescence assay (Pierce Chemical Company, Rockford, IL, USA) and evaluated by densitometry. The sample loading was verified by immunostaining of smooth muscle β-actin.

2.6. Real-time polymerase chain reaction (PCR)

Total RNA was isolated from frozen aortic tissue using TriPure Isolation Reagent (Roche, Mannheim, Germany) according to the protocol of the manufacturer. Integrity of total RNA was evaluated by agarose gel electrophoresis. Total RNA concentration and purity were determined by measuring absorbance at 260 and 280 nm (NanoDrop 2000; Thermo Scientific, Waltham, MA, USA). Reverse transcription (RT) was performed using random hexamers primers, 4 μg of total RNA as template in 50-μl reaction volume and the High-Capacity cDNA RT Archive Kit (Applied Biosystems, Carlsbad, CA, USA). Aortic gene expression of iNOS and TNF-α was determined by quantitative real-time PCR (qRT-PCR) using commercial TaqMan probes. mRNAs for eNOS and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits (NOX-1, $p47^{phox}$ and $p22^{phox}$) were quantified by qRT-PCR based on SYBR Green fluorescence using a LightCycler 480 II PCR system (Roche, Spain) [21]. Expression levels of cDNA were compared to the internal standard β-actin to normalize the data. The specific primer sequences were the following: β-actin (Rn00667869, Applied Biosystems), iNOS (Rn00561646, Applied Biosystems), TNF-α (Rn00562055_m1, Applied Biosystems), eNOS (forward 5′-TGTCACTATGGCAACCAGCGT-3′ and reverse 5′-GCGCAATGTGAGTCCGAAAA-3′), NOX-1 (forward 5′-CCTTCCATAAGCTGGTGGCAT-3′ and reverse 5′-GCCATGGATCCCTAAGCAGAT-3′), $p22^{phox}$ (forward: 5′-GGCCATTGCCAGTGTGATCTA-3′ and reverse 5′-TGCTTGATGGTGCCTCCAA-3′) and $p47^{phox}$ (forward 5′-AGGAGATGTTCCCCATTGAGG-3′ and reverse 5′-CAGTCCCATGAGGCTGTTGAA-3′). Threshold cycle (Ct) values obtained for each gene were referenced to β-actin (ΔCt) and converted to the linear form using $2^{-\Delta Ct}$ as a value directly proportional to the copy number of cDNA and initial quantity of mRNA [21].

2.7. In situ detection of superoxide anion

The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate production of arterial $O_2^{\bullet-}$ *in situ* as previously described [19]. Fourteen-micrometer-thick aortic sections were placed on gelatin-coated slides and equilibrated for 30 min at 37°C in Krebs-HEPES buffer (in mmol/L: NaCl 130, KCl 5.6, $CaCl_2$ 2, $MgCl_2$ 0.24, HEPES 8.3, glucose 11) (pH 7.4). Tissue sections were incubated in HEPES-buffered solution containing DHE (2 μmol/L) (546-nm excitation and 610-nm emission) in a light-protected humidified chamber (37°C, 30 min). Preparations were viewed by laser scanning confocal microscope (TCS SP2; Leica, Heidelberg, Germany; ×16 objective) using the same imaging settings in each case. In preliminary experiments, DHE fluorescence was almost abolished by the $O_2^{\bullet-}$ scavenger PEG-superoxide dismutase, indicating the specificity of this reaction.

For quantification, integrated optical densities were calculated from four sampled areas per ring for each experimental condition using MetaMorph Image Analysis Software (Molecular Devices, Sunnyvale, CA, USA).

2.8. Immunofluorescence

Transverse sections (14 μm) of aortic rings were placed on gelatin-coated slides and air-dried. After blockade, sections were incubated with a polyclonal antibody against iNOS (1:100; Thermo Scientific) in 2% bovine serum albumin–PBS in a humidifier chamber (37°C, 1 h). Afterwards, preparations were incubated with the secondary antibody, a donkey anti-rabbit (1:200) IgG conjugated to Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Immunofluorescent signals were viewed using an inverted Leica TCS SP2 confocal laser scanning microscope (×16). Cy3-labeled antibody was visualized by excitation at 568 nm and detection at 600 to 700 nm. The specificity of the immunostaining was evaluated by omission of the primary antibody followed by the same protocol as above. Under these conditions, no staining was observed in the vessel wall in any experimental situation.

Optical density of immunofluorescence was assayed with MetaMorph Image Analysis Software (Molecular Devices). Four areas per ring were sampled for each experimental condition. The integrated optical densities in the target region were calculated.

2.9. Drugs and chemicals

All chemicals were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). 1400W was supplied by Tocris Bioscience (Bristol, UK). All drugs were prepared in distilled water.

2.10. Expression of results and statistical analysis

Data represented are means±S.E.M. of $n=7$ rats. Vasoconstrictor responses were expressed in g/mg of dry tissue. Vasodilatation was expressed as a percentage of the previous tone generated by Phe. Two-way analysis of variance followed by Bonferroni's comparison test was used to compare the results. Differences were considered significant when $P<.05$. A Prism GraphPad Software 5.0 (San Diego, CA, USA) was used for statistical analysis.

3. Results

3.1. Effect of RBEE on vascular reactivity

3.1.1. Vasodilatation

To evaluate endothelial function, endothelium-dependent vasodilatation to ACh was examined in aortas from the experimental groups. ACh induced concentration-dependent relaxation that was significantly reduced in aortic rings from OC rats compared to lean rats (Fig. 1A). The endothelial dysfunction in OC was also revealed by a lower value of pEC_{50} ($-\log EC_{50}$) when comparing curves to ACh (pEC_{50} OC: 7.25±0.06, LC: 7.86±0.24; $P<.05$), without altering maximal relaxing responses. RBEE significantly restored this impairment in endothelial-dependent vasodilatation to ACh in aorta from obese Zucker rats (Fig. 1A). This restoration was particularly evident in rats treated with the lowest concentration of RBEE (O1%), reaching similar levels of vasodilatation to those observed in lean rats (Fig. 1A) (pEC_{50}: 7.58±0.06, $P<.05$). Endothelium-independent vasodilatation induced by the NO donor SNP in endothelium-denuded aortic rings did not differ significantly between the experimental groups (Fig. 1B).

Aortas from lean Zucker rats treated with RBEE-enriched diet did not show significant changes in endothelium-dependent and -independent dilatation compared to nontreated lean rats (Supplementary Figure 1 (A–B)).

The contribution of NO in the vasodilatation to ACh was also analyzed. Inhibition of NOS with L-NAME evoked a marked reduction in endothelium-dependent vasodilatation for all the experimental groups, and this attenuation was even more evident in aorta from obese animals (Fig. 2A). To evaluate the contribution of vascular iNOS in this effect, we used the selective iNOS inhibitor 1400W. Vasodilatation curves to ACh in the presence of 1400W (Fig. 2B) revealed a marked implication of iNOS in the vasodilator response to ACh in aortic rings from OC compared to LC. This contribution was significantly attenuated by treatment with 1% RBEE. Representation of the differences between the area under the curves in the presence of L-NAME (nonselective inhibition of NOS isoforms) and in the presence of 1400W (selective inhibition of iNOS) revealed the participation of eNOS in vasodilatation in each experimental group (Fig. 2C). In this sense, aortas from OC exhibited a decreased contribution of eNOS — and therefore an enhanced iNOS implication — that was significantly restored by RBEE in a concentration-dependent manner (Fig. 2C).

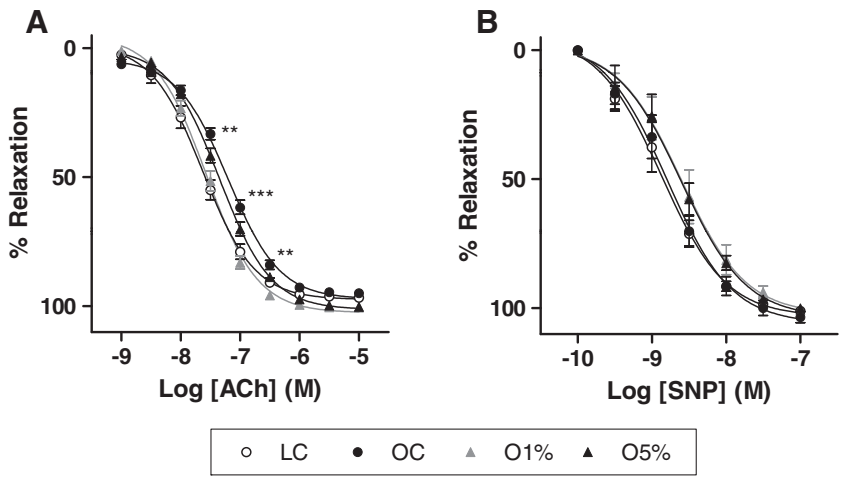

Fig. 1. Concentration–response curves to ACh (A) or SNP (B) to evaluate endothelium dependence or independence of the vasodilatation, respectively. Data are mean±S.E.M. ($n=7$). **$P<.01$, ***$P<.001$ vs LC.

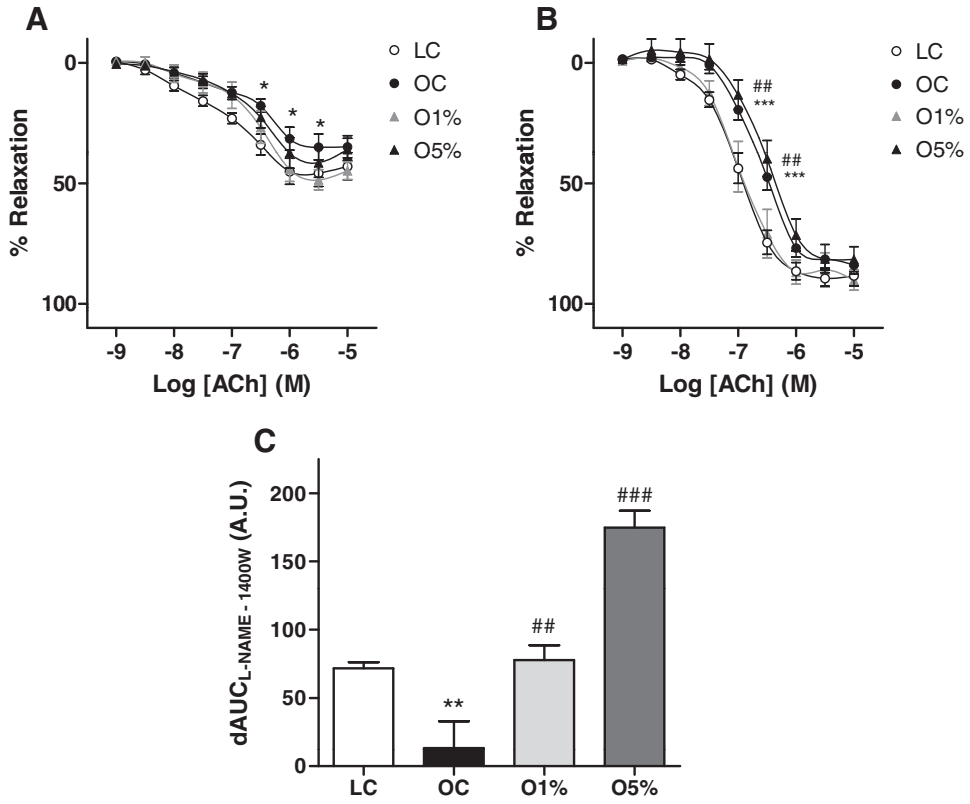

Fig. 2. Concentration–response curves to ACh in the presence of a nonselective NOS inhibitor, L-NAME (A), or a selective iNOS inhibitor, 1400W (B). Differential areas under the curves (dAUCs) obtained from cumulative curves to ACh in the presence of L-NAME and 1400W were graphically represented (C). dAUCs were expressed as arbitrary units (AU). Data are mean±S.E.M. (n=5–7). *P<.05, **P<.01, ***P<.001 vs LC; ##P<.01, ###P<.001 vs OC.

3.1.2. Vasoconstriction

Endothelium-intact aortic rings from OC rats exhibited an increased contractile response to Phe in comparison to LC (Fig. 3A). Treatment with RBEE significantly attenuated this hyperreactivity to Phe in obese animals (Fig. 3A). This attenuation was especially evident in the O1% group, showing a similar pattern of contraction to Phe than that reached in LC (Fig. 3A). Removal of endothelium enhanced vasoconstriction in all experimental groups when comparing to contractile curves in aortas with functional endothelium (Fig. 3B, P<.05). The attenuation of the hyperreactivity to Phe induced by 1% RBEE in intact aortic rings was significantly reversed by endothelium removal (Fig. 3B). RBEE treatment did not modify Phe responses in aortas from lean Zucker rats (Supplementary Figure 1 (C–D)).

The presence of either L-NAME or 1400W enhanced contractile responses to Phe in all experimental groups compared to intact aortic rings in the absence of the inhibitor (Fig. 3C and D, P<.05). Under these conditions, OC rats still showed hyperreactivity to Phe in comparison to LC that remained unaltered by the presence of either L-NAME or 1400W (Fig. 3C and D). On the other hand, O1% group exhibited a marked attenuation of the concentration–response curve to Phe after NOS inhibition (Fig. 3C and D), especially when iNOS was selectively inhibited. Regarding O5%, vasoconstriction was not altered by L-NAME, whereas selective inhibition of iNOS with 1400W induced a significant attenuation Phe response when compared to intact aortic rings (Fig. 3D, P<.05).

3.2. Effects of RBEE on vascular eNOS

Aortas from lean and nontreated obese Zucker rats exhibited similar levels of eNOS protein expression (Fig. 4A). However, aortas from RBEE-treated animals exhibited a significant up-regulation of eNOS expression, especially in the 5% RBEE-treated group (Fig. 4A). Regarding aortic mRNA levels of eNOS, no differences were appreciated between the experimental groups, indicating a posttranscriptional regulation of eNOS (Fig. 4B).

3.3. Effects of RBEE on vascular iNOS and TNF-α

Aortic tissue from OC animals showed an increased protein expression of iNOS, particularly in the smooth muscle layer, whereas no immunostaining was found in arterial preparations from lean rats (Fig. 5A). Treatment with RBEE induced a marked attenuation of aortic iNOS immunofluorescence in the vascular wall (Fig. 5A). Quantification of the fluorescence signals (Fig. 5B) evidenced the significant inhibition of iNOS aortic immunofluorescence by RBEE in obese animals. According to these results, analysis of mRNA levels of vascular iNOS revealed a substantial increase in OC compared to LC and a significant attenuation in obese groups treated with RBEE (Fig. 5C). Therefore, RBEE decreased vascular induction of iNOS in obese Zucker rats at both mRNA and protein level.

Fig. 5D shows vascular expression of TNF-α mRNA in the experimental groups. Expression levels of the inflammatory cytokine were significantly increased in nontreated obese rats compared to LC, whereas the O1% and O5% groups revealed a marked reduction of aortic mRNA levels of TNF-α (Fig. 5D).

3.4. Effects of RBEE on vascular superoxide anion production and NADPH oxidase

To characterize and localize vascular $O_2^{\bullet-}$ production, aortic sections were exposed to DHE, which is oxidized by $O_2^{\bullet-}$ to yield

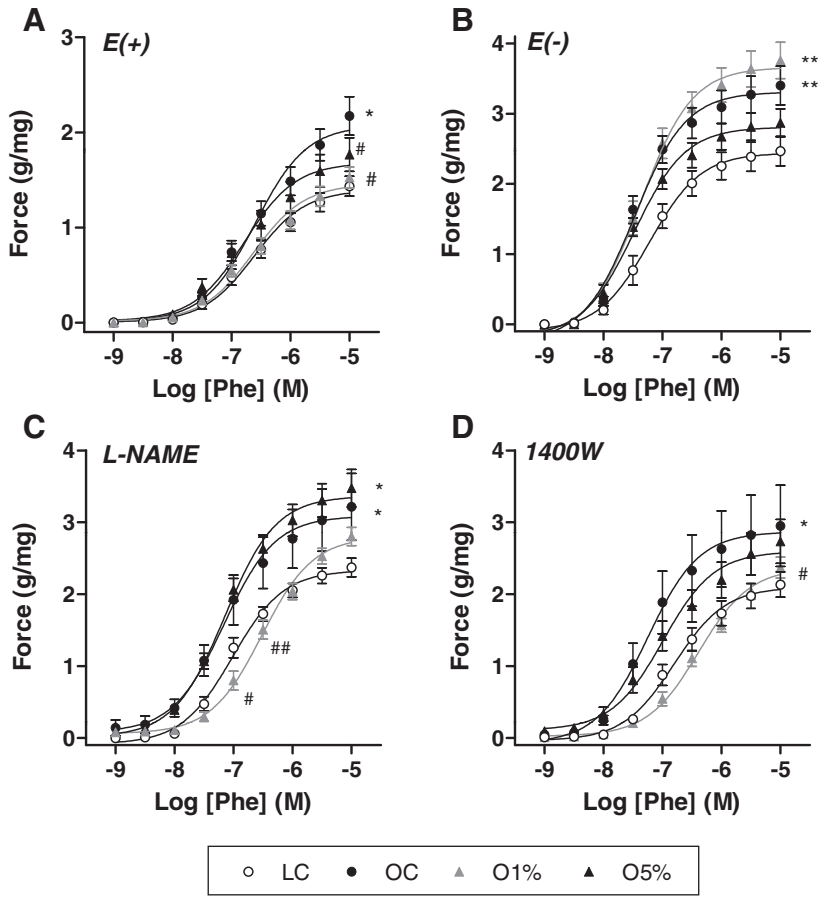

Fig. 3. Concentration–response curves to Phe in endothelium-intact (A) and endothelium-denuded (B) aortic rings in the absence of inhibitors or in intact aortas exposed to a nonselective NOS inhibitor, L-NAME (C), or a selective iNOS inhibitor, 1400W (D). Data are mean±S.E.M. (n=5–7). *P<.05, **P<.01 vs LC; #P<.05, ##P<.01 vs OC.

the red fluorescent DNA stain ethidium. Aortic sections from OC rats showed marked increase staining throughout the vessel wall when compared to LC (Fig. 6A and B). Red fluorescence from all vascular cell layers was significantly reduced in aortic preparations from obese rats treated with RBEE (Fig. 6A and B).

Analysis of mRNA levels of NADPH oxidase subunits, which are the major source of vascular $O_2^{\bullet -}$, showed augmented mRNA levels of NOX-1 and $p22^{phox}$ subunits in OC aortic rings compared with LC, whereas arterial segments from 1% and 5% RBEE-treated animals evidenced a significant reduction of these values (Fig. 6C and D). However, no significant differences were observed when comparing mRNA levels of $p47^{phox}$ subunit (Fig. 6E).

4. Discussion

Several studies support the beneficial properties of rice bran extracts in cardiometabolic risk factors such as dyslipidemia, hypertension and glucose metabolism [22,23]. Recent investigations of our group have evidenced the beneficial effects of a novel water-soluble RBEE *in vivo* on animal models of hypercholesterolemia [24] and metabolic syndrome [16]. Particularly, obese animals fed an RBEE diet showed an important improvement on lipid profile, blood pressure levels, insulin resistance and plasmatic levels of inflammatory biomarkers compared to nontreated obese rats [16]. The present investigation has found the beneficial effect of RBEE on vascular and endothelial dysfunction associated with obesity and the main mechanisms involved in this activity.

Vascular abnormalities related to obesity, in particular the impaired vasodilatation in various vascular beds and in response to different stimuli, might affect vascular homeostasis, peripheral vascular resistance and the delivery of substrates to metabolically active tissues, thereby contributing to hypertension, atherosclerosis and metabolic disorders [5]. One of the most characteristic vascular alterations in metabolic syndrome is the endothelial dysfunction, which seems to involve a reduction in the amount of the available endothelium-derived NO within the vasculature. Indeed, patients with metabolic syndrome secondary to central obesity show impaired forearm vasodilator response to ACh [25]. Regarding our results, a moderate grade of endothelial dysfunction was developed by obese Zucker rats that was restored by RBEE-supplemented diet, especially by 1% RBEE. The fact that aortas from RBEE-treated obese rats showed up-regulation of eNOS protein expression, but not at mRNA levels, compared to nontreated animals suggests that RBEE could induce a posttranscriptional activation of eNOS and hence increase endothelial NO production, which is the main mediator of vasodilatation in conductance arteries such as aorta. However, it is important to take into account that eNOS expression does not reflect enzyme activity. In line with these results, the use of nonselective and selective inhibitors of iNOS revealed the potential participation of eNOS to vasodilatation in aortas from RBEE-treated obese rats.

Another vascular alteration present in obese Zucker rats is vascular hyperreactivity, evidenced by an increased vascular tone. This hyperresponse has been associated with an increased myogenic activation [26], an elevated expression and activity of serotonin [27] and endothelin receptors [28], as well as an increased sympathetic

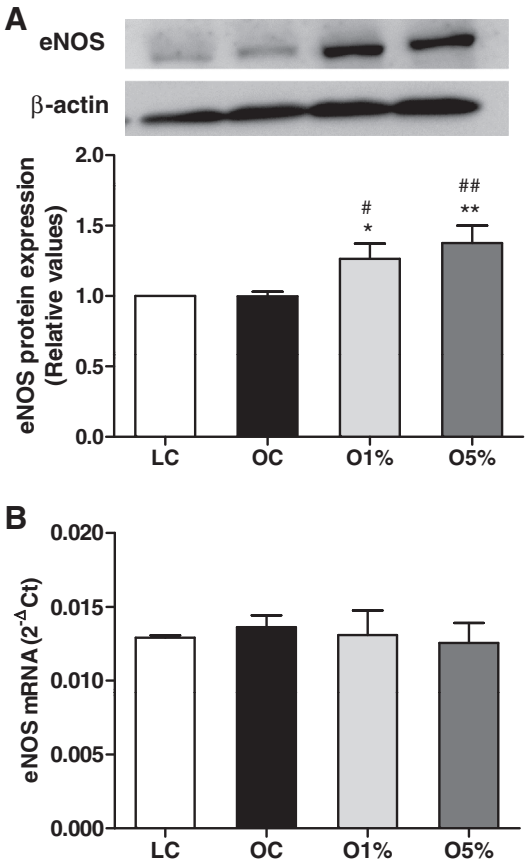

Fig. 4. eNOS expression in aorta from Zucker rats. (A) Immunoblots (top) of aortas probed with antibodies against eNOS or β-actin, as indicated. Graph (down) shows the optical densitometric analysis of pooled data. (B) eNOS mRNA expression in aorta normalized to the expression of the endogenous reference gene β-actin. Data are mean ±S.E.M. (n=5–7). *P<.05, **P<.01 vs LC; #P<.05, ##P<.01 vs OC.

nervous system activity [29]. In the development of obesity, an increased adrenergic activity is frequently observed [30], which can have a profound impact on the perfusion of tissues which are sensitive to adrenergic modulation and subsequently on blood pressure levels [31]. In the present study, RBEE treatment ameliorated hypercontractility to Phe, reaching a vasoconstriction profile similar to that found in lean rats. This response had an important endothelium-dependent component and revealed a different grade of participation of NOS isoforms. The up-regulation of eNOS evidenced in RBEE-treated groups compared to nontreated rats might be involved in the attenuation of vascular vasoconstriction to Phe by RBEE in intact aortic segments. In addition, the use of nonselective and selective inhibitors of iNOS revealed an important contribution of this isoform in aortic hyperreactivity of obese Zucker rats. Treatment with RBEE seemed to attenuate iNOS involvement, reaching similar levels of participation as observed in lean rats. This finding suggests the potential action of RBEE on vascular inflammation related to obesity, which will be discussed later. We could also suggest that the effect of RBEE on vasoconstriction, joined with its direct effects on vascular endothelium, might be involved in the attenuation of blood pressure levels previously evidenced in obese Zucker rats treated with this rice bran extract [16].

On the other hand, it is known that obesity is characterized by iNOS overexpression in the vasculature [32], as confirmed by our results. The excessive NO production from iNOS has been shown to reduce insulin-mediated glucose uptake and induce cellular stress and pancreatic β-cell death in obese animals [32]. For this reason, the implication of this enzyme as an obesity-associated inflammation biomarker was evaluated. Selective inhibition of iNOS by 1400W confirmed the important involvement of this isoform in the vascular reactivity of obese Zucker rats, which was modified by the RBEE-supplemented diet. Indeed, while obese animals showed augmented values of iNOS aortic protein expression and mRNA levels compared to LC, these parameters were significantly restored in aortas from RBEE-treated animals. Therefore, the key role developed by NO from vascular iNOS was significantly ameliorated by treatment with a diet supplemented in RBEE. These results are in accordance with our previous investigation in which obese rats treated with RBEE showed an important attenuation of the proinflammatory plasmatic levels of nitrates and nitrites when compared to nontreated obese animals [16].

Accordingly, it has been demonstrated that the best characterized adipocytokine causing insulin resistance and endothelial dysfunction in metabolic syndrome is TNF-α, a proinflammatory molecule which under normal circumstances circulates in low concentrations [33]. Increased expression levels of iNOS are strongly related to an up-regulation of TNF-α [34], as we have found in aorta from obese Zucker rats. In this regard, vascular expression of TNF-α was reduced with both concentrations of RBEE in accordance to iNOS down-regulation and plasmatic adiponectin restoration (Supplementary Table I and Ref. [16]). The attenuation of the vascular proinflammatory state associated with obesity agrees with previous studies where a diet supplemented in γ-oryzanol and other bioactive components of rice bran improved levels of different inflammatory biomarkers in obese subjects [35].

Vascular oxidative stress plays a key role in the vascular impairment associated with metabolic syndrome. In general, treatment of obese Zucker rats with antioxidants has improved vasodilator responses to NO-dependent stimuli in isolated microvessels and using *in situ* preparations [36], suggesting that vascular reactivity can be improved by acute treatment with oxidative radical scavengers in this animal model. In this regard, an elevated antioxidant capacity has been attributed to rice bran because of its high content in phenolic compounds [37,38]. Besides, it has been demonstrated that γ-oryzanol and related compounds have antioxidant and ROS scavenging potency, inhibiting proinflammatory cytokines activity and increasing blood adiponectin levels *in vitro* and *in vivo* [13]. Antioxidant activity of γ -oryzanol has been attributed to its ferulic acid moiety [39] as well as to the sterol moiety in living cells [40]. In our study, the content of RBEE in these bioactive components could play a critical role in the antioxidant action induced by RBEE in the vascular wall of obese Zucker rats.

The NADPH oxidase system is a major source of vascular ROS [41]. It has been suggested that rice bran constituents may interfere with the NADPH oxidase system, which in turn inhibits ROS production [13]. Several studies support the important role of NADPH oxidase-derived ROS in hypertension and atherosclerosis using animal models with genetic disruption of subunits of the enzyme [42,43]. When evaluating the antioxidant effects of RBEE in the vascular tissue, another target in our investigation was to determine aortic expression levels of NADPH subunits. It was found a reduction in RBEE-treated aortas in the expression of the subunits NOX-1 and $p22^{phox}$, which were importantly increased in obese animals, contributing to vascular dysfunction and partially to hypertension. Vascular ROS generation may be the upstream target of RBEE followed by the improvement of vascular reactivity and inflammatory biomarkers.

In summary, this investigation demonstrates that chronic administration of this novel water-soluble enzymatic extract of rice bran could be a suitable treatment for improving or alleviating vascular alterations associated with metabolic syndrome. RBEE-supplemented diet ameliorates vascular impairment related to obesity by restoring endothelial dysfunction and

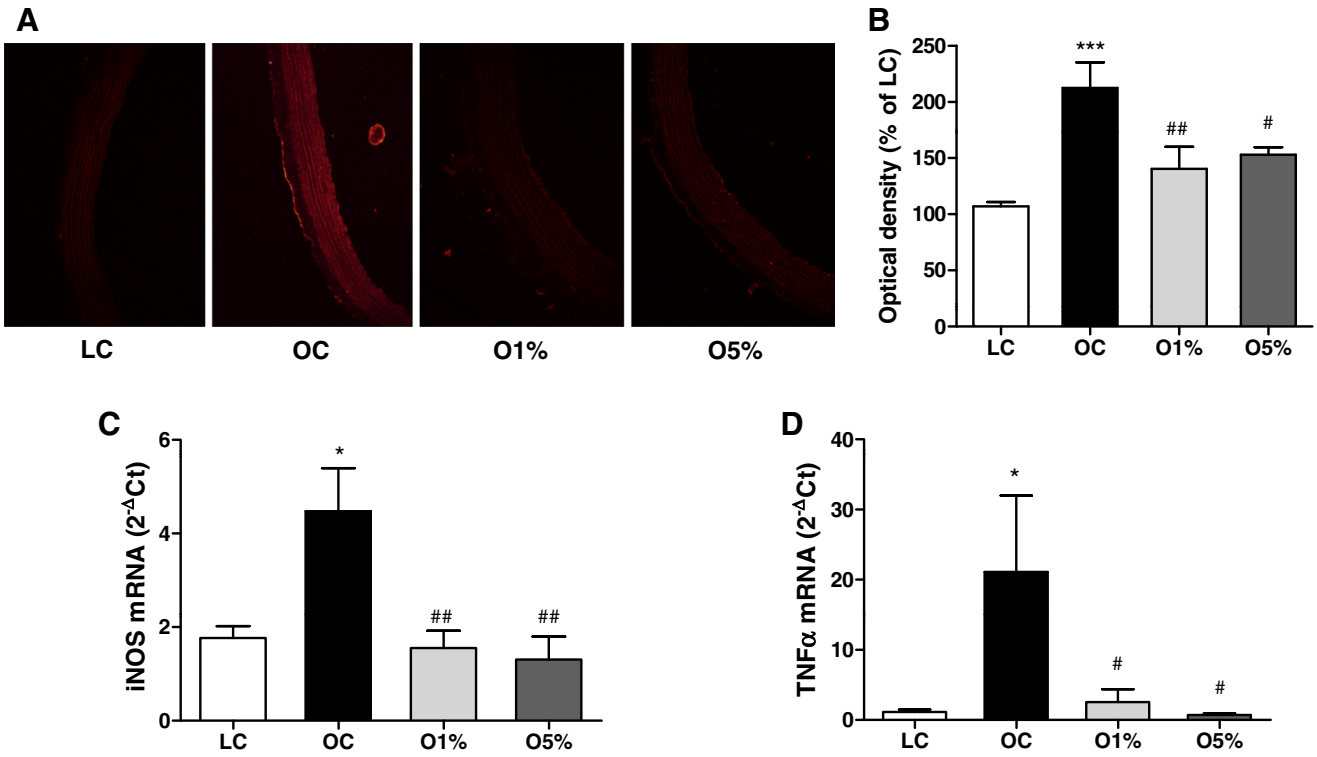

Fig. 5. iNOS protein expression by immunofluorescence (A,B) and mRNA levels of iNOS (C) and TNF-α (D) in aorta. Data are mean±S.E.M. (n=5–7). $^{*}P$<.05, $^{***}P$<.001 vs LC; $^{\#}P$<.05, $^{\#\#}P$<.01 vs OC.

vascular hyperreactivity via a counteraction of iNOS and TNF-α involvement probably in association with a notable reduction of vascular oxidative stress (Fig. 7). According to the present study and our previous investigations [16], the beneficial effect of RBEE on vascular alterations linked to metabolic syndrome seems to be a consequence of a reduction in vascular, adipose and plasmatic

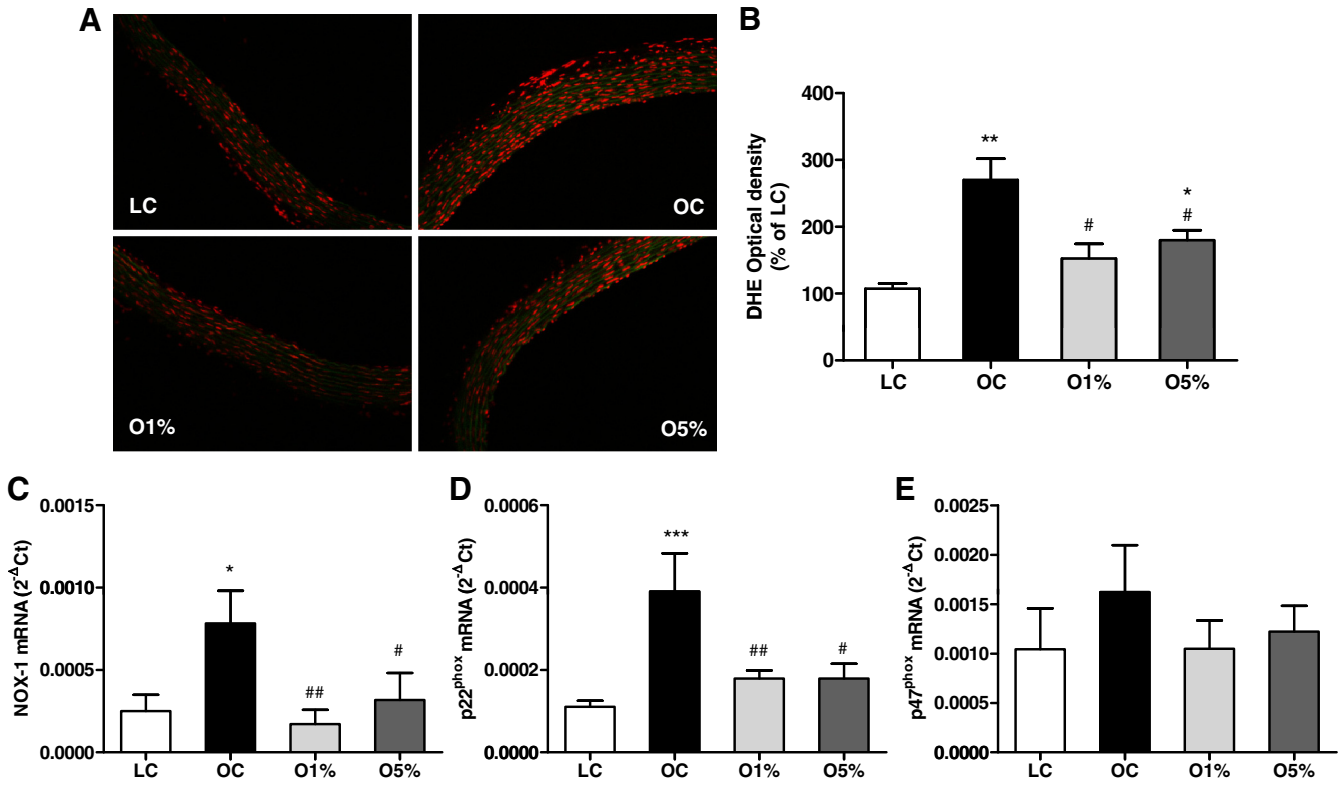

Fig. 6. Aortic production of superoxide anion by DHE staining (A, B) and mRNA levels of the NADPH oxidase subunits NOX-1 (C), $p22^{phox}$ (D) and $p47^{phox}$ (E). Data are mean±S.E.M. (n=5–7). $^{*}P$<.05, $^{***}P$<.001 vs LC; $^{\#}P$<.05, $^{\#\#}P$<.01 vs OC.

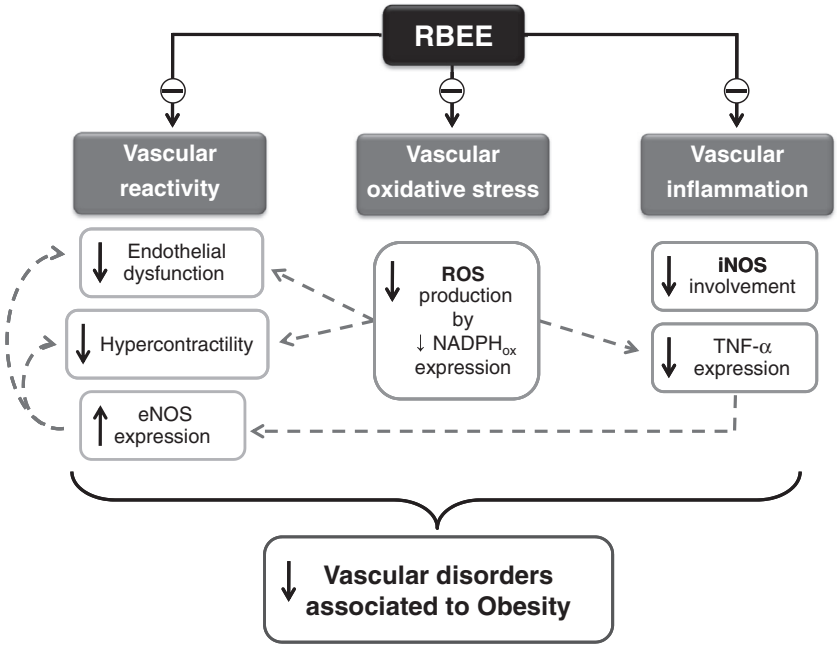

Fig. 7. Schematic representation of mechanisms involved in the beneficial effects of RBEE in obesity-related vascular impairment. RBEE treatment attenuates vascular ROS production. This effect is related to a restoration of vascular reactivity by reducing endothelial dysfunction and hypercontractility as well as up-regulating eNOS expression. Also, RBEE attenuates vascular inflammation by reducing iNOS and TNF-α involvement, thus contributing to the improvement of vascular disorders associated with obesity. NADPHox, nicotinamide adenine dinucleotide phosphate-oxidase.

proinflammatory and pro-oxidants biomarkers joined with an improvement in lipid profile and insulin resistance. These findings support the idea that RBEE could be considered an exemplary candidate to be used as functional food in the treatment of vascular complications associated with obesity.

Acknowledgments

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Appendix A

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2012.12.004.

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ARTÍCULO 3: **Microvascular disorders in obese Zucker rats are restored by a rice bran diet**

Nutrition, Metabolism & Cardiovascular Diseases (2014)

Desórdenes de los microvasos en ratas Zucker obesas son restaurados por una dieta de salvado de arroz

Justo *et al,* 2014. *Nutrition, Metabolism & Cardiovascular Diseases*

En el estudio anterior se ponen de manifiesto los efectos beneficiosos que ha mostrado tener la inclusión de EESA en la dieta sobre la hipertensión moderada y las alteraciones de los vasos de conductancia asociadas al síndrome metabólico [84]. Teniendo en cuenta que el deterioro de la función de los vasos de resistencia de pequeño calibre es un factor de riesgo detectable de forma temprana [87], el objetivo de este estudio fue clarificar si una dieta suplementada con EESA era capaz de atenuar las alteraciones producidas en los microvasos de ratas Zucker obesas.

Para ello, durante 20 semanas se les administró una dieta estándar enriquecida al 1% y 5% de EESA a ratas Zucker obesas (O) y sus controles delgadas (L), estableciéndose cuatro grupos de tratamiento (L1%, L5%, O1% y O5%), además de sus grupos controles alimentados sólo con dieta estándar (LC y OC). Se realizaron estudios de funcionalidad en anillos de arterias mesentéricas de segundo orden, los cuales se montaron en un miógrafo isométrico de alambres. Se evaluó la vasodilatación dependiente de endotelio mediante curvas acumulativas de acetilcolina, las cuales se hicieron en presencia o ausencia de inhibidores como L-NAME, INDO, apamina y caribdotoxina para estudiar la implicación del óxido nítrico, los derivados de la COX, y el factor hiperpolarizante dependiente de endotelio (EDHF), respectivamente. Paralelamente, se determinaron las variaciones de la expresión vascular de SK_{Ca}, IK_{Ca} y eNOS por *Western Blot*, y de iNOS por inmunofuorescencia. Además, se determinó la producción vascular de aniones superóxido mediante la detección de fluorescencia de dihidroetidio.

Como resultado, el tratamiento prolongado con una dieta suplementada con EESA restauró la función de los microvasos en los animales obesos tratados. Esto se confirma con la elevada implicación de óxido nítrico y EDHF que denota el significativo aumento que se produjo en la expresión de eNOS y de los canales de calcio dependientes de

potasio, respectivamente. Por otro lado, en los animales obesos controles tuvo lugar un incremento de la expresión de iNOS en la pared vascular y de presencia de anión superóxido, parámetros ambos que fueron atenuados de forma significativa en los animales obesos tratados con EESA 1% y 5%.

A la luz de estos datos, podemos decir en primer lugar que EESA es capaz de restaurar la función vascular a distintos niveles, ya que además de atenuar la disfunción de arterias de conductancia en los animales obesos, también fue capaz de reducirla en los microvasos mediante la recuperación de la implicación de mediadores como el óxido nítrico y los canales de potasio dependientes de Ca^{2+} implicados en la contribución del EDHF. Esto, junto con los estudios previos, nos invita a pensar que este extracto actúa de forma multi-factorial y positiva en diferentes puntos de la patología cardiovascular que rodea al síndrome metabólico, poniendo de manifiesto este estudio su utilidad en la prevención de las enfermedades cardiovasculares por su efecto sobre los microvasos. Esta versatilidad terapéutica convierte a EESA en una interesante alternativa a la terapia farmacológica convencional que se aplica a las complicaciones asociadas a la obesidad, siendo a su vez una buena estrategia preventiva en términos de costes de salud e impacto socio-económico, debido a la accesibilidad que tiene la materia prima de la que se obtiene, a la rentabilidad de su extracción y su fácil administración.

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Microvascular disorders in obese Zucker rats are restored by a rice bran diet

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KEYWORDS
Rice bran;
Mesenteric artery;
Obese Zucker rat;
Microvascular function;
Obesity

Abstract *Background and aim:* Nutritional-based approaches aimed to prevent microvascular dysfunction associated to obesity present potential advantages over pharmacological strategies. Our aim was to test whether a rice bran enzymatic extract (RBEE)-supplemented diet could attenuate microvascular alterations in obese rats.
Methods and results: Lean and obese Zucker rats were fed standard diet supplemented or not with 1% and 5% RBEE for 20 weeks. Functional studies were performed in small mesenteric arteries in isometric myograph. Immunoblotting and fluorescence studies were made in arterial homogenates and arterial sections, respectively. RBEE-supplementation restored microvascular function in obese rats through a marked increase in NO and endothelial-derived hyperpolarizing factor contribution by up-regulation of eNOS and calcium-activated potassium channels expression, respectively, in association to a substantial reduction of microvascular inflammation and superoxide anion formation. These data agrees with the beneficial actions of RBEE on dyslipidemia, hyperinsulinemia and hypertension in obesity.
Conclusion: The multi-factorial properties of RBEE-diet, especially for restoring the function of small resistance arteries shows this dietary-based approach to be a promising candidate for prevention of microvascular alterations in obesity, which are crucial in cardiovascular events in obese subjects.

Introduction

Obesity has detrimental effects in the vasomotor function of arteries, especially in the microcirculation [1–5]. Although one of the most common pharmacological approaches in clinical complications of obesity is the control of many aspects of diabetes, these therapies inadequately prevent atherothrombosis and microvascular disorders in spite of the important health costs that these therapies involve [6,7]. As an alternative to pharmacological approaches, numerous studies position diet food or dietary components with functional properties as first strategy in the prevention of obesity-related disorders, principally for their potential advantages (over pharmacological treatments of obesity and its complications) in terms of health costs and socio-economical impact [7–10]. Particularly, recent evidences have found an important relationship between diet and macro- and microvascular alterations in obesity [1,4,11].

In this sense, rice, and particularly rice bran, is an excellent nutritional source of bioactive compounds, including high healthy value proteins and phytochemicals (i.e. γ-oryzanol and ferulic acid) with potent antioxidant, hypolipidemic and anti-inflammatory activities [12]. The therapeutic use of rice bran is limited because of the insolubility of its proteins, the integrity of its nutraceutical compounds, and the rancidity of its oil-derived. These

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limitations have been counteracted by the production of a water-soluble rice bran enzymatic extract (RBEE) [13–15] that provides numerous advantages over other rice bran derivatives regarding water solubility, increased content in nutraceutical compounds and lack of rancidity. Our recent investigations have evidenced that a diet supplemented with RBEE is able to ameliorate cardiometabolic risk factors in obese Zucker rats, showing a remarkable action on dyslipidemia, hypertension, insulin resistance and adipokines production [14,15]. In particular, RBEE-enriched diet restored vascular impairment related to obesity in conductance arteries by decreasing endothelial dysfunction and vascular hyperreactivity via a counteraction of iNOS and TNF-α involvement in association to a notably reduction of aortic oxidative stress [15].

In the present study we evaluate the potential beneficial effect of a dietary intervention in microvascular disorders associated to obesity, with particular emphasis on the effect of RBEE-supplemented diet on the contribution of endothelial-derived factors, such as nitric oxide (NO) and endothelial derived hyperpolarizing factor (EDHF), to the vasodilator response of small mesenteric arteries from obese Zucker rats. Elucidation of these actions in resistance arteries might contribute to the characterization of the microvascular dysfunction in obesity as well as to the effect of dietary approaches supplemented in RBEE in cardiovascular and metabolic parameters of obese animals found in our previous investigations.

Methods

Animals, dietary intervention and biochemical parameters

RBEE was prepared and characterized as previously described [13] (Supplementary Tables S.I, S.II and S.III). Briefly, protein was the major component (38%). The fat components present in RBEE (30%) were mainly soluble because of protein interactions. Minor functional components of lipid fraction in RBEE included phytosterols (4084 mg/kg), γ-oryzanol (1260 mg/kg), tocopherols (99 mg/kg) and tocotrienols (174 mg/kg). The enzymatic treatment increased concentrations of protein and minor functional components, especially γ-oryzanol and tocols, which were more than three-fold higher compared to the original raw material RB [13].

Obese Zucker rats and their littermate controls, lean Zucker rats (8 weeks aged, Charles River Laboratories, Barcelona, Spain) were fed standard diet and water *ad libitum*. Obese rats were divided into three groups ($n = 7$) and daily treated with either 1% RBEE (O1%), 5% RBEE supplementation (O5%), or standard diet (Obese control group, OC). A group of lean Zucker rats ($n = 7$) was also fed a standard diet (Lean control group, LC). RBEE treatment was administered during 20 weeks as a syrup form included in standard diet, supplemented with the concentrations indicated above [14,15]. Nutritional relevance of the selected RBEE concentrations are based on previous studies using effective concentrations of rice bran extracts and γ-oryzanol in either humans [16,17] or animal models [18,19].

Body weight, food and water intake, and systolic blood pressure were weekly evaluated. At the end of treatment, animals were anesthetized and blood samples were collected by intracardiac puncture for biochemical assays in serum (Table 1), as described [14,15]. The protocol for animal handling and experimentation agreed with the European Union European Community guidelines for the ethical treatment of animals (EEEC Directive of 1986; 86/609/EEC) and was approved by the Ethical Committee for Animal Research of the University of Seville.

Vascular reactivity

Segments of small mesenteric arteries (SMA) were mounted in wire-myographs filled with Krebs–Henseleit solution (KHS). Mechanical activity was measured isometrically by a force transducer Myograph System-610M (DMT, Aarhus, Denmark) coupled to a Powerlab data acquisition system (AD-Instruments, Australia) [20].

Table 1 Body weight, blood pressure and serum parameters and inflammatory cytokines of the experimental groups.

	LC	OC	O1%	O5%
Body weight (g)	461 ± 11^{a}	569 ± 21^{c}	606 ± 27^{c}	601 ± 29^{c}
Systolic blood pressure (mmHg)	124 ± 3^{a}	162 ± 2^{b}	152 ± 3^{c}	139 ± 2^{a}
Serum parameters:				
TC (mmol/L)	2.35 ± 0.10^{a}	6.0 ± 0.3^{b}	5.4 ± 0.2^{c}	5.0 ± 0.7^{d}
HDL-C (mmol/L)	0.88 ± 0.07^{a}	2.4 ± 0.3^{b}	2.9 ± 0.2^{c}	3.2 ± 0.3^{d}
TG (mmol/L)	0.98 ± 0.15^{a}	4.6 ± 0.6^{b}	4.0 ± 0.2^{c}	3.30 ± 0.15^{d}
Glucose (mmol/L)	6.6 ± 0.6^{a}	8.7 ± 1.0^{c}	9.4 ± 0.5^{c}	9.0 ± 1.0^{c}
Insulin (ng/mL)	0.48 ± 0.12^{a}	4.5 ± 0.6^{b}	4.1 ± 0.5^{c}	2.6 ± 0.6^{d}
Adiponectin (ng/mL)	13.7 ± 0.3^{a}	7.7 ± 0.8^{b}	13.9 ± 1.6^{a}	19.5 ± 3^{a}
$NO_{(x)}$ (μmol/L)	6.4 ± 0.3^{a}	52 ± 10^{b}	36 ± 5^{c}	21 ± 5^{d}
Inflammatory cytokines (VAT) (relative mRNA):				
TNF-α ($2^{-\Delta}$Ct)	1.0 ± 0.07^{a}	2.59 ± 0.39^{b}	0.56 ± 0.22^{c}	0.60 ± 0.14^{c}
IL-6 ($2^{-\Delta}$Ct)	1.0 ± 0.41^{a}	1.67 ± 0.31^{b}	0.45 ± 0.18^{c}	0.52 ± 0.2^{c}
IL-1β ($2^{-\Delta}$Ct)	1.0 ± 0.4^{a}	0.84 ± 0.27^{a}	0.09 ± 0.09^{b}	0.12 ± 0.10^{b}

LC, lean controls fed standard diet (SD); OC, obese controls fed SD; O1, obese fed 1% RBEE-supplemented diet; O5, obese fed 5% RBEE-supplemented diet. Values in a row without a common superscript letter differ significantly ($P < 0.05$). *VAT*: *visceral adipose tissue*.

Endothelium-dependent vasodilatation to acetylcholine (ACh) (1 nmol/L to 10 μmol/L) was studied in intact SMA pre-contracted with phenylephrine (1 μmol/L). To evaluate the participation of the different components in endothelium-dependent vasodilatation, concentration–response curves to ACh were performed in the presence of the following drugs (alone or in combination): the non-selective cyclooxygenase (COX) inhibitor indomethacin (INDO, 10 μmol/L), the NO synthase inhibitor N^{ω}-Nitro-L-arginine methyl ester (L-NAME, 300 μmol/L), the small-conductance calcium-activated potassium (SK_{Ca}) channel blocker apamin (Apa, 100 nmol/L) and the blocker of both SK_{Ca} and intermediate-conductance K_{Ca} (IK_{Ca}) channels charybdotoxin (Ctx, 50 nmol/L). These K_{Ca} channels are the main mediators of the EDHF response.

Immunoblotting and immunofluorescence

Immunoblotting of homogenized tissue [15] was performed using the specific primary antibodies: anti-SK_{Ca}, anti-IK_{Ca} (1:1000; Santa Cruz Biotech, Heidelberg, Germany) and anti-eNOS (1:800; Cell Signaling Technology, MA, USA) followed by the secondary anti-rabbit antibody (Dako, Denmark). Blots were developed using chemiluminescence and densitometric analysis of the specific bands was performed by ImageJ software (USA). The sample loading was verified by immunostaining of β-actin.

For immunofluorescence studies [15], SMA sections (14 μm) were incubated with a polyclonal antibody against iNOS (1:100; Thermo Scientific, MA, USA) followed by the secondary antibody, a donkey anti-rabbit (1:200) IgG conjugated to Cy3 (Jackson ImmunoResearch Laboratories, West Grove, USA). Signals were viewed using a laser scanning confocal microscope (TCS SP2; Leica, Heidelberg, Germany; ×40 objective). Optical density of immunofluorescence was assayed with MetaMorph Image Analysis Software (Molecular Devices, Sunnyvale, CA, USA).

In situ detection of superoxide anion

The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate production of arterial $O_2^{\cdot-}$ *in situ*. 14-μm-thick SMA sections placed on gelatin-coated slides were incubated with DHE (2 μmol/L) in Krebs-HEPES buffer [15]. Preparations were viewed by laser scanning confocal microscope (TCS SP2; Leica, Heidelberg, Germany; ×40 objective). In these experiments, DHE fluorescence was abolished by the $O_2^{\cdot-}$ scavenger, PEG-superoxide dismutase, indicating the specificity of this reaction. Integrated optical densities were quantified using MetaMorph Image Analysis Software (Molecular Devices, Sunnyvale, CA, USA).

Results expression and statistical analysis

Data represented are means ± SEM. Vasodilatation was expressed as a percentage of the previous tone generated by phenylephrine. One- or Two-way ANOVA followed by Bonferroni's comparison test was used to compare the results, as appropriate. Differences were considered significant when $P < 0.05$. A Prism GraphPad Software 5.0 (San Diego, CA, USA) was used for statistical analysis.

Results

Basic parameters and inflammatory biomarkers of the animals used

Obese Zucker rats weighted significantly more than their lean controls. Dietary intervention with RBEE did not significantly modify the overweight in obese animals (Table 1). RBEE-diets attenuated hypercholesterolemia in obese animals and improved levels of HDL-cholesterol in a dose-dependent manner. Although hyperglycemia in obese groups remained unchanged, rats fed RBEE-enriched diets showed attenuated levels of hyperinsulinemia characteristic in obesity. In addition, high values of systolic blood pressure in obese rats were significantly attenuated by RBEE administration. When analyzing inflammatory biomarkers, RBEE improved levels of plasmatic adiponectin whereas the expression of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) from adipose tissue as well as plasmatic levels of nitrates/nitrites ($NO_{(x)}$) were significantly reduced (Table 1).

Vascular reactivity

To evaluate endothelial function, endothelium-dependent vasodilatation to ACh was assessed in SMA segments. ACh-induced response was the same in all four groups (Supplementary Fig. S.1A). Similarly, when analyzing the accumulative contractile response to phenylephrine, no changes were observed between the experimental groups (Supplementary Fig. S.1B).

To further investigate the endothelial-derived components that could be affected by either obesity or RBEE treatment in the ACh-induced vasodilatation, we examined the effect of different pharmacological inhibitors. The contribution of the EDH-component in SMA was analyzed by simultaneous inhibition of COX-derived factors and NO synthesis by the presence of INDO and L-NAME, respectively. In these conditions, SMA from non-treated obese rats showed an important impairment in EDH-contribution compared to lean rats, and this contribution was significantly restored by RBEE-supplemented diets (Fig. 1A and B). Blockage of COX with INDO and EDH with the K_{Ca} channel inhibitors Apa and Ctx, revealed NO contribution in ACh response. Participation of NO in vasodilatation was significantly lower in resistance arteries from obese rats. Although this impairment remained unchanged in the O1% group, arteries from obese rats fed a 5% RBEE diet showed an increased NO contribution at the same extent as that in lean rats (Fig. 1C and D). Finally, the presence of L-NAME, Apa and Ctx at the same time evidenced a very low contribution of COX-derived products in vasodilatation to ACh in SMA, which was similar for all four experimental groups (Fig. 1E and F).

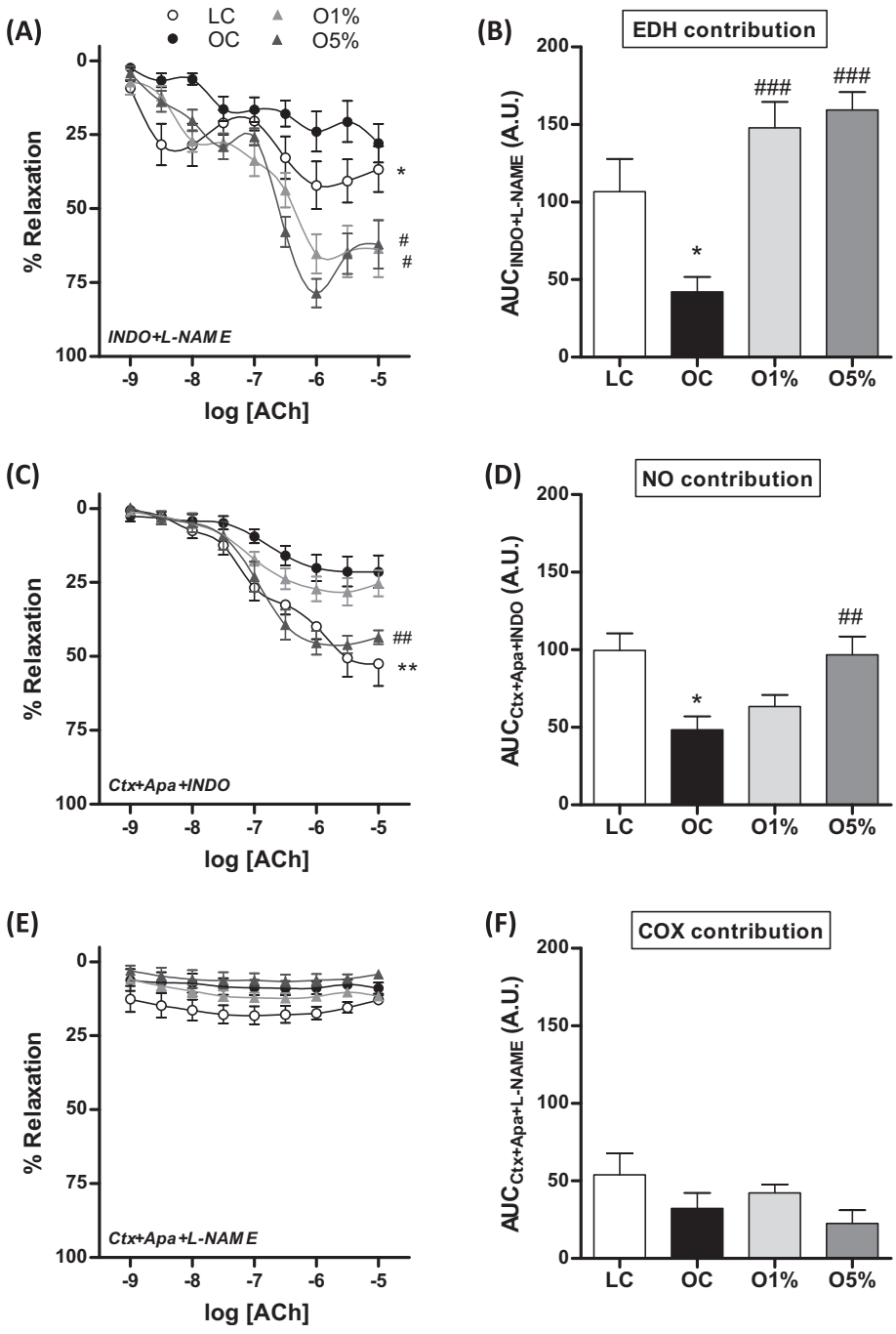

Figure 1 Concentration-response curves to acetylcholine (ACh) (A, C, E) in the presence of the combination of inhibitors of NO synthase (L-NAME), COX (indomethacin, INDO) and EDH response (Charybdotoxin and apamin; Ctx and Apa, respectively). Areas under the curve (AUC) obtained from every cumulative curve to ACh in the presence of the combinations of the inhibitors were graphically represented (B, D, F). AUC were expressed as arbitrary units (A.U.). Data are mean ± S.E.M. (n = 5–7). $^{*}P < 0.05$, $^{**}P < 0.05$ vs LC. $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ vs OC.

K_{Ca} channels and eNOS expression

SMA from obese rats exhibited a significant decrease in the expression of both SK_{Ca} and IK_{Ca} (Fig. 2A and B). Administration of RBEE-enriched diets induced a marked increase in protein expression of SK_{Ca} (Fig. 2A). IK_{Ca} expression was only restored by diets enriched in 5% RBEE (Fig. 2B). Protein expression of eNOS was also ameliorated in SMA from obese Zucker rats and this was significantly up-regulated by a 5% RBEE administration (Fig. 2C).

iNOS immunofluorescence

SMA sections from obese control rats showed an increased protein expression of iNOS, particularly in the smooth muscle cells, when compared to lean groups (Fig. 2D). Treatment

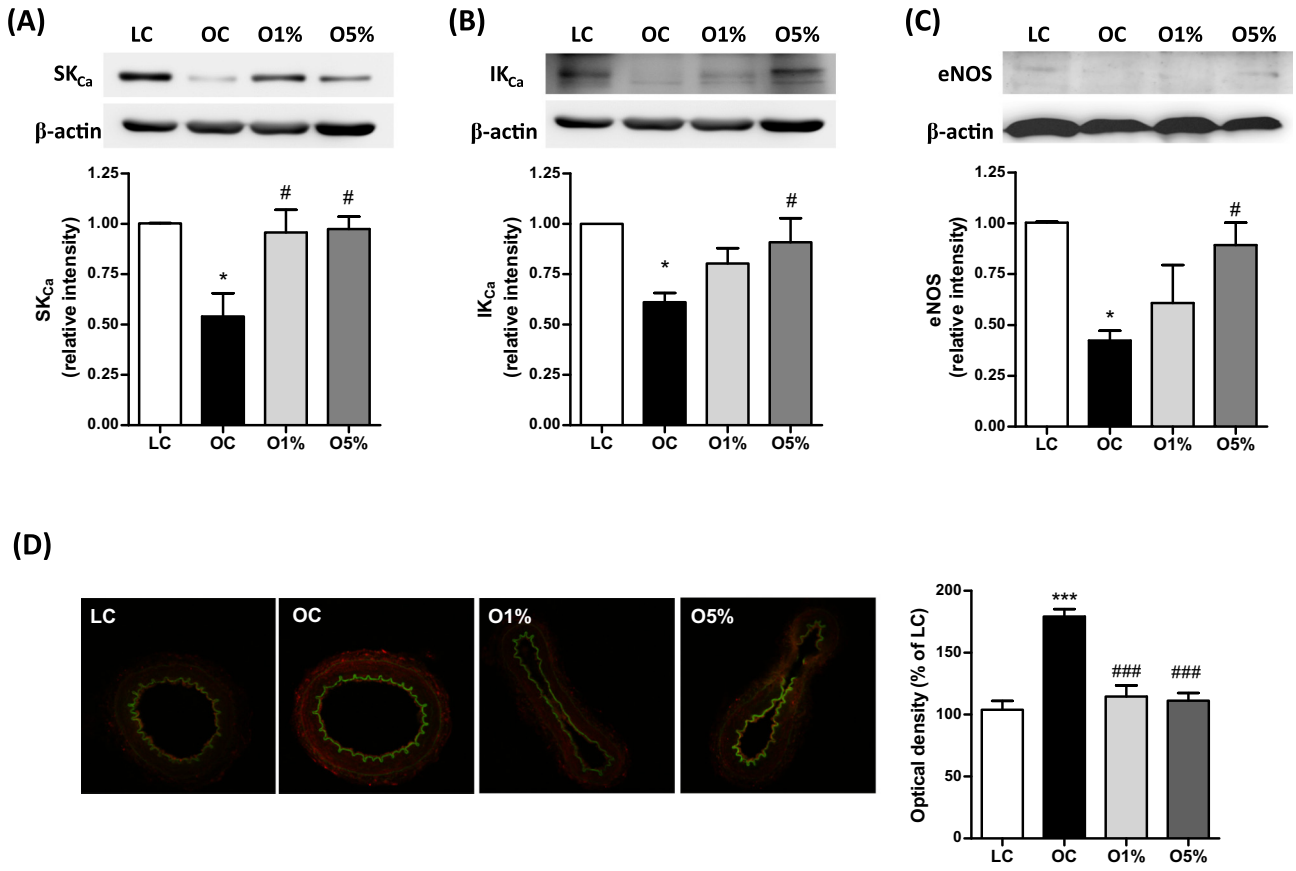

Figure 2 Expression of small and intermediate conductance calcium-activated potassium channels (SK_{Ca} and IK_{Ca}, respectively), endothelial NO synthase (eNOS) and inducible NO synthase (iNOS) in small mesenteric arteries from Zucker rats. Western blots revealing the expression of SK_{Ca} (A), IK_{Ca} (B) and eNOS (C). Results were quantified by densitometry and presented as the ratio of density of SK_{Ca}, IK_{Ca} and eNOS bands vs that of β-actin from the sample (A–C). Immunofluorescence images showing iNOS expression in arterial sections and results of optical density quantification (D). Data are mean ± S.E.M. ($n = 3–5$). $^{*}P < 0.05$ vs LC. $\#P < 0.05$ vs OC.

with RBEE induced an important attenuation of arterial iNOS immunofluorescence in the vascular wall (Fig. 2D).

Superoxide anion production

To investigate vascular $O_2^{\cdot-}$ production, SMA sections were exposed to DHE, which is oxidized by $O_2^{\cdot-}$ to yield the red fluorescent DNA stain ethidium. SMA from obese rats showed a marked increase staining throughout the vessel wall compared to the lean control group (Fig. 3). Red fluorescence from all vascular cell layers was significantly reduced in mesenteric preparations from obese animals treated with RBEE (Fig. 3).

Discussion

This study demonstrates that administration of a rice bran enriched-diet attenuates microvascular disorders in obesity through a marked restoration on the contribution of endothelium-derived mediators to the arterial function in association to a substantial reduction of inflammation and oxidative stress in SMA from obese Zucker rats. These results are in agreement with our observations showing the ability of rice bran supplementation to ameliorate disorders related to metabolic syndrome such as dyslipidemia, insulin resistance, hypertension and vascular impairment of large conductance arteries [14,15].

Microvascular dysfunction may affect both peripheral vascular resistance and insulin-mediated glucose disposal, thereby contributing to hypertension and insulin resistance, respectively, in obesity [3]. Although several studies in humans and animal models have evidenced the beneficial daily intake of rice bran and other wholegrain derivatives in metabolic disorders associated to obesity and diabetes [15–19,21], their specific effects on microvascular complications linked to these disorders remain unknown. In our investigation we have evidenced for the first time the beneficial effect of a rice bran diet administration on obesity-related alterations in small resistance arteries.

Several studies have shown a blunted vasorelaxation in obese subjects, with a reduced contribution of NO [22] and EDHF [23]. In our study, although endothelial-dependent dilatation to ACh was similar between the experimental groups, there was an evident impaired NO contribution to the vasodilatation in SMA from obese rats. We observed that arteries from obese animals treated with 5% RBEE showed an improved vasorelaxation similar to the lean control group in the presence of the NO-independent inhibitors. This fact could be related to the up-regulation that RBEE administration induced in eNOS vascular

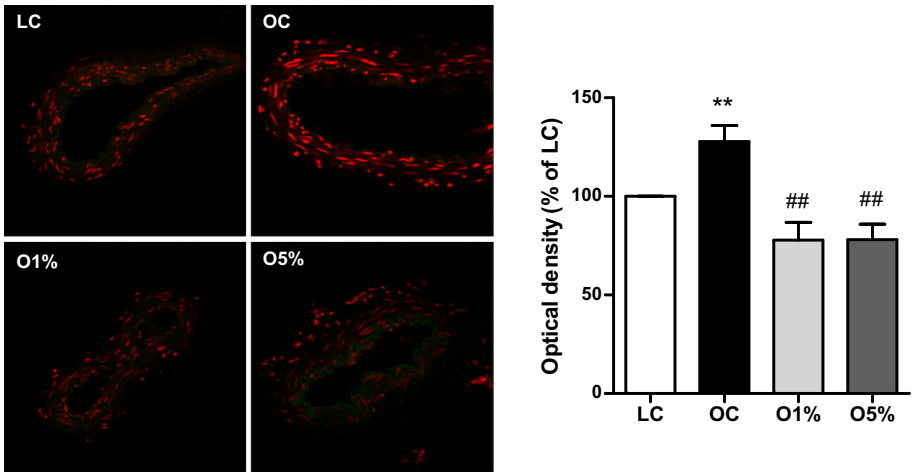

Figure 3 Superoxide anion production by fluorescence DHE staining in sections of small mesenteric arteries from Zucker rats. Graph (right) showing optical density of DHE fluorescence. Data are mean ± S.E.M. ($n = 5$). $^{**}P < 0.01$ vs LC. $\#\#P < 0.01$ vs OC.

expression. Our results agree with previous investigations where, despite the lack of changes in vasodilatation to ACh between lean and obese Zucker rats, there was a reduced inhibitory effect in the dilatation of SMA from obese rats after simultaneous inhibition of COX and EDHF-responses compared to lean rats [11].

Experimental studies suggest that the contribution of EDHF increases as the vessel size decreases, with predominant activity in resistance arteries and a compensatory up-regulation of EDHF in states characterized by reduced NO availability [24]. For this reason, the restored EDHF contribution observed in SMA from obese animals fed RBEE-supplemented diets could be an alternative resource to counteract the loss of NO availability in these animals. EDHF has been shown particularly important in the microcirculation, where systemic vascular resistance is regulated [24,25]. Despite the on-going dispute over its identity, it is generally accepted that EDHF-dependent dilatation depends on endothelial SK_{Ca} channels [25,26]. Several authors have described how functional SK_{Ca} channels are down-regulated in resistance arteries from obese animals [4,23,27], as observed in the present investigation. This EDHF impairment has been related to the metabolic disorders characteristic in obese Zucker rats, especially glucose and insulin alterations [23,27]. In our study, a significant increase in SK_{Ca} expression was induced by RBEE-enriched diets in obese animals compared to the obese control group, reaching similar expression levels as the lean group. Also, 5% RBEE induced an upward trend in both SK_{Ca} and IK_{Ca} expression. These results could be associated to the beneficial effects of RBEE in insulin resistance demonstrated in obese Zucker rats [14]. Also, the effect of RBEE-diets on NO and EDHF contribution could contribute to the improvement in systolic blood pressure levels observed in obese Zucker rats fed rice bran diets by an enhanced peripheral vascular function [5,27].

Many lines of evidence suggest that increased vascular oxidative stress plays an important role in endothelial dysfunction and, in turn, in the impairment of NO/EDHF-mediated relaxation and the development of cardiovascular disease [11,26]. Vascular depolarization leads to $O_2^{\cdot-}$ production and NO bioavailability reduction [28]. Blockade of SK_{Ca} and IK_{Ca} converts the ACh-induced hyperpolarization into depolarization, evidencing the role of these channels in the control of ROS signaling in vascular cells [28]. RBEE-enriched diet induces a significant attenuation of $O_2^{\cdot-}$ production in SMA from obese Zucker rats that could be closely linked to the significant restoration of K_{Ca} channels and EDHF contribution induced by this dietary supplement.

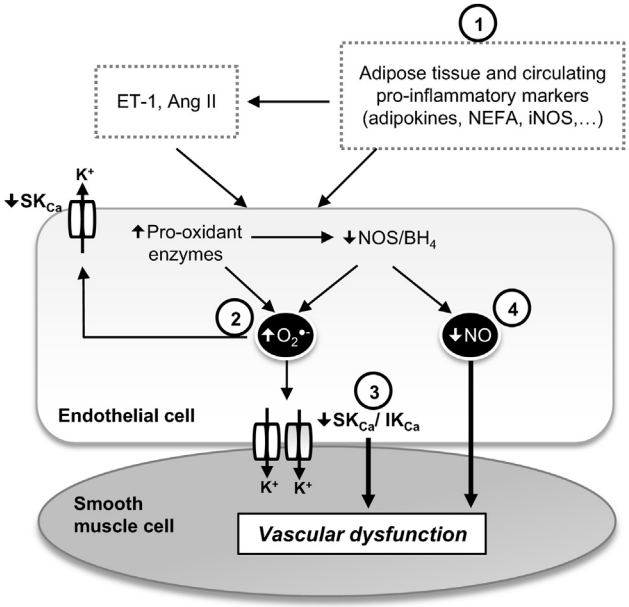

Figure 4 Representation of the mechanisms involved in the effects of RBEE in the dysfunction of small resistance arteries in obesity. Administration of RBEE attenuates inflammatory markers from adipose tissue (1) with the subsequent attenuation in vascular superoxide anion (2), increased levels of NO (3) and the restoration of calcium-activated potassium channels activation to maintain the EDH-response in the vasodilatation (4).

In obesity exists an increased production of pro-inflammatory cytokines released from adipose tissue, such as tumor necrosis factor (TNF)-α and interleukin-6, and a decreased production of the anti-inflammatory adipokine adiponectin [29]. Abdominal obesity is closely associated with microvascular dysfunction, through indirect mechanisms, such as insulin-resistance, and directly, because of the production by the perivascular adipose tissue of adipokines and pro-inflammatory cytokines which in turns induce oxidative stress [1,3,30]. Enhanced inflammatory markers in obesity are not limited to adipose tissue and circulating blood, but also to the vascular wall. In this sense, obese Zucker rats present increased levels of pro-inflammatory markers such as TNF-α and iNOS expression in visceral adipose tissue and aorta [15,29]. Also, plasmatic levels of adiponectin are attenuated in these obese rats [14]. Administration of RBEE-enriched diet restored levels of these inflammatory indicators in both adipose tissue and aorta from obese Zucker rats [15]. In the present investigation, resistance arteries from obese rats fed a RBEE diet showed decreased levels of iNOS expression, suggesting the anti-inflammatory potential of this treatment in obesity. The beneficial effect of RBEE against inflammation in adipose tissue might be related to the improved profile of inflammatory indicators in microvessels.

To summarize, RBEE-supplementation was able to restore microvascular function in obese Zucker rats, by increasing the EDHF contribution throughout up-regulation of endothelial SK_{Ca} and IK_{Ca} channels and a partial increase of NO bioavailability from eNOS (Fig. 4), both mechanisms as a compensatory response against metabolic and vascular disorders in obesity. This positive effect was associated to a significant reduction of superoxide production and iNOS expression in the vascular wall. The beneficial effects of RBEE on inflammatory markers from visceral adipose tissue could play a critical role on these microvascular responses (Fig. 4). The management of obesity-related clinical disorders including microvascular dysfunction is currently based on the treatment of their individual components, e.g. hypertension or insulin resistance. Due to the multi-factorial properties demonstrated by RBEE, especially restoring function of small resistance arteries, this dietary-based approach may be a promising candidate for prevention of cardiovascular and metabolic complications of obesity. Particularly, the present investigation provides evidence for the beneficial effects of rice bran supplementation in the prevention of microvascular alterations associated to obesity and the mechanisms mediating these actions, involving potential advantages over pharmacological strategies to treat obesity-related complications in terms of health costs and socio-economical impact.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.numecd.2013.10.032

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ARTÍCULO 4: **Rice bran enzymatic extract supplemented diets modulate adipose tissue inflammation markers in Zucker rats**

Nutrition (2014)

Dietas suplementadas con un extracto enzimático de salvado de arroz modulan marcadores de inflamación en el tejido adiposo de ratas Zucker

Candiracci and Justo *et al,* 2014. *Nutrition*

El estado de obesidad se caracteriza, entre otros síntomas, por cursar con un proceso pro-inflamatorio a nivel sistémico, el cual, va muy unido al desarrollo de insulino-resistencia y diabetes cuando estamos ante una obesidad avanzada [3]. Esto se manifiesta en una serie de cambios que se dan en la morfología y funcionalidad del tejido adiposo, donde se da una expansión del mismo a la vez que una hipertrofia de los adipocitos. Descubrirlo como tejido dinámico y responsable de la secreción de moléculas de gran importancia en el proceso inflamatorio como son el TNF-α o las interleuquinas pro- y anti-inflamatorias, lo ha convertido en una diana terapéutica de gran interés [89]. Se sabe que la inflamación moderada asociada al síndrome metabólico es consecuencia de la acumulación de macrófagos en la grasa blanca, y a su vez, de la desregulación en la liberación de adipoquinas. Por tanto, el objetivo de este estudio fue evaluar si la administración de una dieta suplementada con EESA podía atenuar el estado pro-inflamatorio que se daba como consecuencia de las alteraciones del tejido adiposo visceral que tenía lugar en ratas Zucker obesas.

Para ello, durante 20 semanas se les administró una dieta estándar enriquecida al 1% y 5% de EESA a ratas Zucker obesas (OB), estableciéndose dos grupos de tratamiento (OB1 y OB5), y otros dos grupos controles de ratas obesas (OBZ) y delgadas (LZ) alimentadas sólo con dieta estándar. Al finalizar el tratamiento, se realizaron cortes histológicos de grasa visceral de dos tipos, abdominal y epididimal, para proceder a la medida del tamaño de los adipocitos. Además, se realizó la extracción de material genético de las muestras de grasa con el fin de determinar los efectos de la dieta en los marcadores más involucrados en el proceso inflamatorio. Esta determinación de la expresión génica se realizó mediante la técnica de la PCR a tiempo real, tanto en muestras de grasa abdominal como epididimal.

Como resultado, se observó cómo los animales obesos tratados con EESA sufrieron cambios significativos en el patrón de distribución de tamaño de sus adipocitos. En el caso de la grasa epididimal, ambas concentraciones de EESA disminuyeron la frecuencia de distribución de los adipocitos de gran tamaño, e incrementaron la cantidad de adipocitos de menor tamaño, tendiendo al perfil de distribución del grupo control delgado. Por otro lado, en la grasa abdominal se observó una disminución significativa en la cantidad de adipocitos de gran tamaño, comparado con el patrón observado en el grupo control obeso. Estos datos demuestran cómo la grasa de los animales tratados con EESA mostraba un perfil en cantidad y tamaño de adipocitos significativamente mejor que el de los animales obesos controles. Respecto a la expresión en el tejido de marcadores de inflamación, se vio disminuida de forma significativa la expresión de TNF-α, iNOS, IL-6, e IL-1β en la grasa abdominal procedente de animales obesos tratados. En el caso de la grasa epididimal, sólo se redujo significativamente la expresión de IL-1β e iNOS en los animales obesos en comparación con sus controles. Los niveles de TNF- α e IL-6 permanecieron inalterados.

Como conclusión, se puede decir que el tratamiento crónico con una dieta suplementada con EESA puede mejorar el síndrome metabólico desarrollado en el modelo Zucker, no sólo a nivel bioquímico, metabólico y recuperando de forma notable su funcionalidad arterial, sino siendo capaz de actuar sobre uno de los puntos críticos de la patología, como es el avance del proceso inflamatorio sistémico asociado a la obesidad.

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Basic nutritional investigation

Rice bran enzymatic extract–supplemented diets modulate adipose tissue inflammation markers in Zucker rats

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ABSTRACT

Objective: Chronic low-grade inflammation in obesity is characterized by macrophage accumulation in white adipose tissue and adipokine production deregulation. Obesity also is characterized by oxidative stress related to inflammatory signaling. The aim of this study was to analyze whether dietary supplementation with a rice bran enzymatic extract (RBEE), rich in bioactive compounds with antioxidant and hypocholesterolemic properties, would ameliorate the inflammatory state existing in visceral adipose tissue of obese Zucker rats.
Methods: Obese Zucker rats and their littermate controls, lean Zucker rats ages 8 wk, were daily fed an enriched diet with either 1% or 5% RBEE supplementation over 20 wk. Measurement of adipocyte size and mRNA expression of proinflammatory molecules from visceral abdominal/epididymal tissue was performed.
Results: An RBEE-supplemented diet decreased the overproduction of tumor necrosis factor-α, interleukin (IL)-6, IL-1 β, and inducible nitric oxide synthase (iNOS), as well as the overproduction of IL-6 and iNOs in visceral abdominal adipose tissue and visceral epididymal adipose tissue, respectively. An RBEE-supplemented diet modified the adipocyte-size distribution pattern in both abdominal and epididymal adipose tissue, shifting it toward smaller cell sizes.
Conclusions: Chronic administration of a novel water-soluble RBEE, rich in polyphenols, tocotrienols and γ-oryzanol, could be a suitable treatment to ameliorate the obesity-associated proinflammatory response.

Introduction

Obesity is characterized by a chronic and systemic low-grade inflammation in adipose tissue that is believed to contribute to the development of insulin resistance (IR) leading to type 2 diabetes and is also a risk factor for cardiovascular diseases (CVDs). This hypothesis has gradually replaced the idea of considering adipose tissue as a simple energy store but also as an endocrine organ that secretes a number of bioactive peptides collectively named adipokines, which are relevant at the interface between the immune and the metabolic systems [1]. The adipose organ includes numerous discrete anatomical depots and although subcutaneous adipose tissues store > 80% of total body, visceral adipose tissue have been shown to correlate better with metabolic syndrome and IR than subcutaneous fat depots [2]. Also, the expression of proinflammatory cytokines is generally higher in visceral than in subcutaneous fat [3,4]. Adipose tissue contains adipocytes as well as fibroblasts, preadipocytes, tissue-resident macrophages, and vascular constituents, being macrophages crucial contributors to inflammation. In fact, inflammation in adipose tissue is partially due to an influx of macrophages that secrete proinflammatory factors like tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1 beta (IL-1 β) and inducible nitric oxide synthase (iNOS) [5–8]. It is known that in obesity there is a remarkable shift in the pool of tissue macrophages from the alternatively activated M2 type to the classically activated M1 type, changing the secretion of cytokines from predominantly anti-inflammatory (M2) to proinflammatory (M1) [9]. In addition to macrophages, adipocytes also secrete

MC and MLJ contributed equally to this work. RR-R and MDH conceived and designed the study. MLJ and MC carried out the experiments. AC conceived the experiments and analyzed the data. All authors were involved in writing the paper and had final approval of the submitted and published versions. The authors declared no conflicts of interest.

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specific cytokines, such as adiponectin and leptin. Adiponectin possesses anti-inflammatory effects and serum levels are markedly decreased in patients with visceral obesity and states of IR such as type 2 diabetes. [1].

Zucker rats have a point mutation resulting in dysfunctional leptin receptors that makes them leptin-insensitive, leading to an altered leptin metabolism and obesity. Thus, these rats are obese, normoglycemic, insulin-resistant, hyperinsulinemic, hypertriglyceridemic, and hypercholesterolemic. Accordingly, the Zucker rat represents an appropriate model for studying the effect of dietary components on factors associated with metabolic disturbances in overweight. Additionally, obese Zucker rats develop a proinflammatory response and oxidative stress [10]. In this context, TNF-α, a proinflammatory cytokine, is overexpressed in obesity and likely mediates IR in animal models of obesity including obese Zucker rats [5,11].

Rice bran, a byproduct of rice milling, is an important source of bioactive molecules of particular interest as an antioxidant and a lipid-lowering compound, including γ-oryzanol, tocols (tocopherols and tocotrienols), and unsaturated fatty acids [12,13]. Although rice bran shows an important composition in natural antioxidants and nutritional proteins [12,14], its potential use as a functional food is limited by the high insolubility of its protein, as well as the integrity of its nutraceutical components, particularly referring to the phenolic fraction. Our group has developed a new product from rice bran, a water-soluble rice bran enzymatic extract (RBEE), which preserves functional properties and improves solubility of proteins and the antioxidant components of rice bran [15]. The enzymatic treatment also increases concentration of minor functional components making our RBEE rich in bioactive compounds as polyphenols, fitosterols, and amino acids with antioxidant [16] and hypocholesterolemic activity [17]. Previous results have already demonstrated that an RBEE-enriched standard diet ameliorated metabolic parameters related to IR and CVD in obese Zucker rats [18,19]. In the light of these data, the main goal of the present study was to investigate the ability of RBEE to prevent the inflammation state present in adipose tissue in obesity, focusing on the putative modulator effects of rice bran extract on cytokine expression in visceral adipose tissue.

Materials and methods

Animals and diets

Obese Zucker (OBZ) rats and their littermate controls, lean Zucker (LZ) rats (8 wk old, Charles River Laboratories, Barcelona, Spain) were fed a standard diet and water ad libitum. OBZ rats were divided into two groups and daily treated with either 1% RBEE supplementation (OBZ1) or 5% RBEE supplementation (OBZ5). Treatment with RBEE was administered over 20 wk as a syrup form included in a standard diet, supplemented with the concentrations indicated above.

RBEE, extracted as previously discussed [15], was supplied by the Enzymatic Production Technology Group of the Department of Biochemistry and Molecular Biology, School of Pharmacy, University of Seville (Spain). The chemical composition of RBEE has been previously characterized [15]. Briefly, protein is the major component (38%) in the form of peptides and free amino acids due to the use of proteases for rice bran stabilization. The fat components present in RBEE (30%) are mainly soluble because of protein interactions. Minor functional components of lipid fraction in RBEE include phytosterols (4084 mg/kg), γ-oryzanol (1260 mg/kg), tocopherols (99 mg/kg), and tocotrienols (174 mg/kg).

Body weight, as well as food and water intake, was weekly evaluated. At the end of treatment, the animals were kept fasting for 12 h and were anesthetized with chloral hydrate 12% intraperitoneally. The animals were then sacrificed, and visceral adipose tissue (abdominal and epididymal) was removed and weighted. Experiments were carried out in accordance with the Guidelines of the European Union Council (86/609/EU), following the Spanish regulations (BOE 67/8509–12, 1988) for the use of laboratory animals and approved by the ethical committee of experimentation of the University of Seville.

Measurement of adipocyte size

Visceral abdominal tissue (VAT) and visceral epididymal tissue (VET) from rats were fixed in paraformaldehyde and embedded in paraffin blocks using conventional histological techniques. Cell area of 1200 to 2800 adipocytes per treatment group (six to eight rats per group) were measured with the image analysis program ImageJ 1.42 software (Bethesda, MD, USA) to determine average cell size and size distribution for each experimental group.

RNA extraction and reverse transcription

For polymerase chain reaction analysis, total RNA from adipose tissue was extracted using the Tripure™ Isolation Reagent (Roche, Germany), according to the instructions of the manufacturer. After isolation, the integrity of the RNA samples was assessed by agarose gel electrophoresis. The yield of total RNA was determined by measuring the absorbance (260/280 nm) and reverse transcription (RT) was performed using random hexamers primers, 4 μg of total RNA as template and the High-Capacity cDNA Archive Kit (Applied Biosystems) following the manufacturer's recommendations as previously described [19].

Real-time PCR

For real-time RT-PCR, each specific gene product was amplified using commercial TaqMan ™ probes using the LighCycler480 sequence detector (Roche, Madrid, Spain) as previously described [19]. The cDNA levels were determined using β-actin as housekeeper. The results were normalized using the β-actin expression. Threshold cycle (Ct) values were calculated using the software supplied by Applied Biosystems.

Protein extraction and immunoblotting

Total proteins were extracted from VAT and VET using Tripure Isolation Reagent (Roche), according to the instructions of the manufacturer and the protein fraction was analyzed.

Immunoblotting was performed as previously described [20]. Primary antibody used was the rabbit polyclonal anti-iNOS (Stressgen-Enzo Life Sciences, Farmingdale, NY, USA. 1:1000.). The bands were visualized by LAS3000 imaging system (Fujifilm). Densitometric analysis of the band corresponding to iNOS was performed by ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). All membranes were reblotted using a monoclonal antibody anti-β-actin (Sigma, MO, USA. 1:10.000) as a loading.

Statistical analysis

Results are presented as means ± SEM unless otherwise indicated. Experiments were performed by analyzing all groups of animals in parallel. Data were analyzed with analysis of variance followed by the Tukey–Kramer test using Graph Pad Prism version 5.00 by Graph Pad Software, Inc. (San Diego, CA, USA). The level of significance was set at a P-value < 0.05.

Results

Body weight and weight of visceral adipose fat

The increase in body weight in OBZ rats was significantly higher ($P < 0.001$) than LZ littermates during the treatment (Table 1). Treatment with RBEE lowered slightly this increases in both groups (OBZ1 and OBZ5).

Table 1
Body weight of animals at day 0 and at the end of the treatment with RBEE-supplemented diet and visceral abdominal (VAT) and epididymal (VET) adipose tissue weight at the end of the treatment with RBEE-supplemented diet

	LZ	OBZ	OBZ1	OBZ5
Body weight (g)				
Initial	240 ± 4.5	296 ± 12.9‡	304 ± 3.7‡	298 ± 5.2‡
Final	463 ± 11.1	617 ± 15.3‡	535 ± 12.6*	560 ± 23,6†
(g/100 g Body weight)				
VAT	1.09 ± 0.10	2.76 ± 0.19‡	2.50 ± 0.12‡	2.09 ± 0.07‡,§
VET	0.80 ± 0.09	1.36 ± 0.22‡	1.51 ± 0.11‡	1.34 ± 0.15‡

Experimental groups: LZ, lean controls fed standard diet; OBZ, obese controls fed standard diet; OBZ1, obese fed 1% RBEE-supplemented diet; OBZ5, obese fed 5% RBEE-supplemented diet. Data are mean ± SEM (n = 5–7). Significant differences: $^{*}P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$ versus LZ, $^{\S}P < 0.01$ versus OBZ.

The weight of abdominal and epididymal fat tissue are evaluated for all experimental groups and are summarized in Table 1. Treatment with RBEE did not alter significantly fat weight in OBZ rats. Only weight of VAT in rats fed a 5% RBEE diet was significantly reduced compared with non-treated obese rats.

Adipocyte size distribution

Sectioned epididymal (Fig. 1A) and visceral (Fig. 2A) adipose tissue showed that the obese control group had significantly larger cells compared with the lean control group. Changes also were observed in the overall size distribution of mature adipocytes from both VET (Fig. 1B, C) and VAT (Fig. 2B, C) when comparing OBZ rats with their LZ littermates. In VET, adipocytes in the OBZ group were nearly double the size compared with the LZ: Lean rats did not show cells > 90 μm, whereas obese rats had some cells > 160 μm (Fig. 1B, C). Regarding to VAT, different size distribution pattern also was observed between obese and lean rats. In LZ, maximal size distribution (42%) was enclosed between 60 and 80 μm and no cells were observed > 120 μm (Fig. 2B, C). Conversely, OBZ showed lower distribution of small size adipocytes and higher frequency of distribution of high size adipocytes, exceeding values > 180 μm (Fig. 2B, C).

Interestingly, RBEE-enriched diets modified the size distribution pattern of obese rats in both VET and VAT, and this modification was even more evident in the shape. VET from OBZ1 and OBZ5 showed an important attenuation (14% of attenuation) in the frequency of distribution in large adipocytes between 80 and 100 μm and an increase (6.8% and 11% of increase, respectively) for smaller adipocytes between 40 and 60 μm compared with the obese control group (Fig. 1B, C). VAT from RBEE-treated rats exhibited a significant attenuation (11% of attenuation) in the frequency of distribution in large adipocytes between 120 and 140 μm (Fig. 2B, C).

mRNA expression of TNF-α

An increase of TNF-α mRNA in VAT depots reaching statistical significance was found in OBZ rats compared with the LZ group (2.6-fold; $P < 0.001$) (Fig. 3A). The daily administration of RBEE for 20 wk reduced the production of this inflammatory cytokine in OBZ1 and OBZ5 groups ($P < 0.001$ with respect to OBZ), restoring its production to lower levels than the LZ group (about twofold less) ($P < 0.001$ OBZ1 with respect to LZ; $P < 0.05$ OBZ5 with respect to LZ).

In VET, the expression of TNF-α was different, and mRNA of this cytokine was not induced in OBZ rats compared with the lean control group (Fig. 4A). The feeding of RBEE did not induce any effect on VET of obese rats.

mRNA expression of IL-6

Our results showed a statistical significant increase of IL-6 mRNA in adipose tissue of the OBZ rats both in VAT and VET compared with the LZ group ($P < 0.001$) (Figs. 3B and 4B). Feeding with RBEE-suplemented diets induced a general decrease of IL-6 mRNA expression. This effect was most evident in abdominal visceral fat than in epididymal tissue. In VAT, there

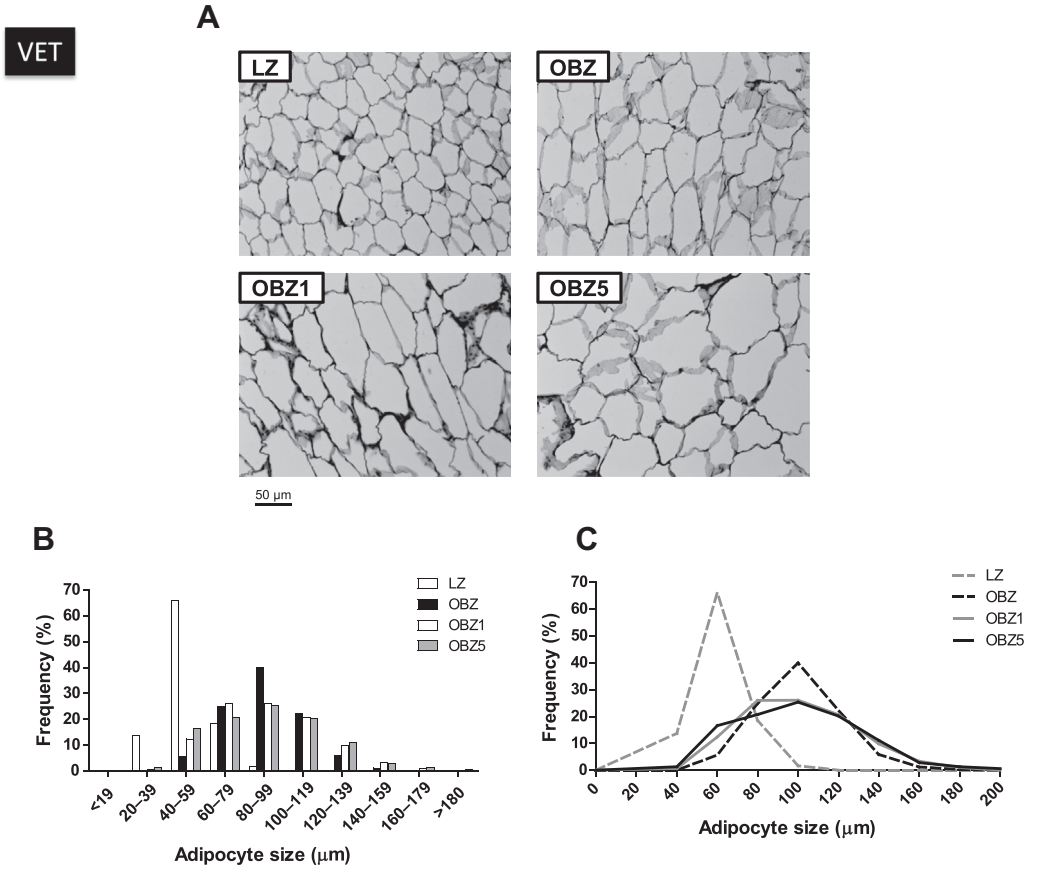

Fig. 1. (A) Histology of epididymal white adipose tissue (VET). (B) and (C) Graphs show distribution (frequency expressed as %) of adipocyte size (μm) in each experimental group. LZ, lean control; OBZ, obese control; OBZ1, obese rats fed 1% RBEE-supplemented diet; OBZ5, obese rats fed 5% RBEE-supplemented diet.

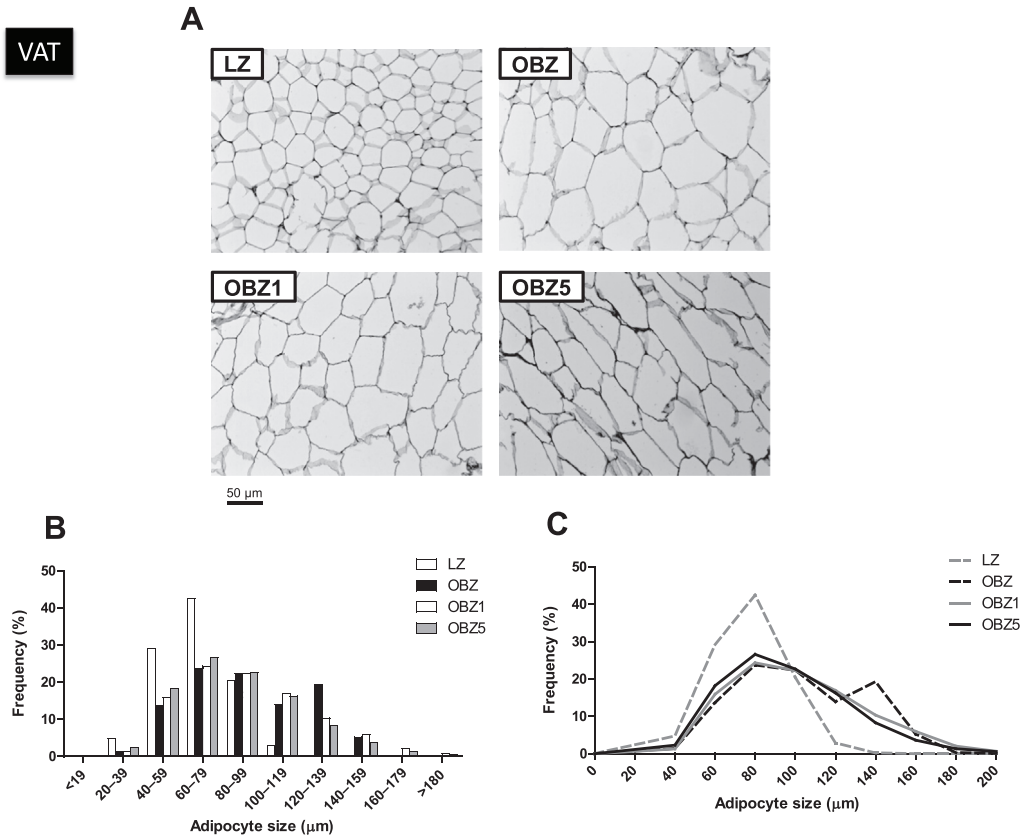

Fig. 2. (A) Histology of abdominal white adipose tissue (VAT). (B) and (C) Graphs show distribution (frecquency expressed as %) of adipocyte size (μm) in each experimental group. LZ, lean control; OBZ, obese control; OBZ1, obese rats fed 1% RBEE-supplemented diet; OBZ5, obese rats fed 5% RBEE-supplemented diet.

was a fourfold decrease of mRNA IL-6 in OBZ1 ($P < 0.001$) and threefold decrease in OBZ5 ($P < 0.001$). These results also were statistically significant with respect to the lean group ($P < 0.01$ OBZ1; $P < 0.05$ OBZ5). In VET only, the 1% RBEE diet was shown to attain statistical significance ($P < 0.001$ compared with OBZ) with a value of IL-6 mRNA similar to the lean group.

mRNA expression of IL-1 β

We did not find significant changes in the expression of IL-1 β mRNA in OBZ rats compared with LZ in both visceral fat tissues examined. However, administration of RBEE reduced the production of the inflammatory cytokine IL-1 β in visceral adipose tissue from obese rats. In VAT, this effect was statistically significant in both RBEE-supplemented diet groups (1% and 5%; $P < 0.001$ in both LZ and OBZ). In VET the decrease of expression of IL-1 β was statistically different only in OBZ1 ($P < 0.01$ OBZ; $P < 0.05$ LZ) (Figs. 3C and 4C).

Expression of iNOS

Our results showed an up-regulation of iNOS at both mRNA (Figs. 3D and 4D) and protein levels (Fig. 5) in both adipose tissues studied compared with LZ rats. RBEE-enriched diets decreased iNOS mRNA and protein expression in both VAT and VET ($P < 0.001$ and $P < 0.05$ in all cases of OBZ; Figs. 3D, 4D, and 5) reaching values lower than in the LZ group ($P < 0.05$ OBZ5 with respect to LZ in VAT; $P < 0.01$ OBZ1 with respect to LZ; $P < 0.05$ OBZ5 with respect to LZ in VET).

Discussion

The purpose of this study was to investigate the effect of a new water-soluble RBEE on inflammatory markers in OBZ rats. Although no significant differences were observed in the final body weight and in the adipose tissue weight between the different groups, RBEE-enriched diets were able to modulate the altered production of cytokines, characteristic of adipose tissue in OBZ rats, and also modified the adipocyte size distribution pattern in both VAT and VET shifting it toward smaller cell sizes.

Obesity is associated with a chronic inflammatory response, which is characterized by abnormal cytokine production, increased synthesis of acute-phase reactants, such as C-reactive protein, and activation of proinflammatory signaling pathways [3]. It has been strongly evidenced that adipokines secreted by the adipose tissue contribute to obesity-associated systemic inflammation and may constitute potential important targets for the prevention of inflammation-induced IR or vasculopathy [1,9]. As a result of overweight and obesity, adipocytes increase in size and release more saturated free fatty acids (FFAs) and chemokines, followed by macrophages infiltration into adipose tissue that leads to an increase in expression of IL-1 β, IL-6, and iNOS [21].

As previously indicated, functional components of the lipid fraction in RBEE include phytosterols, γ-oryzanol, tocopherols, and tocotrienols. Tocotrienols, as well as polyphenols, have proven antioxidant and anti-inflammatory properties and exhibit activity against different chronic diseases, such as cancer, diabetes, CVDs, and neurologic disorders [22,23]. Additionally, γ-tocotrienol has been found to improve obesity-related

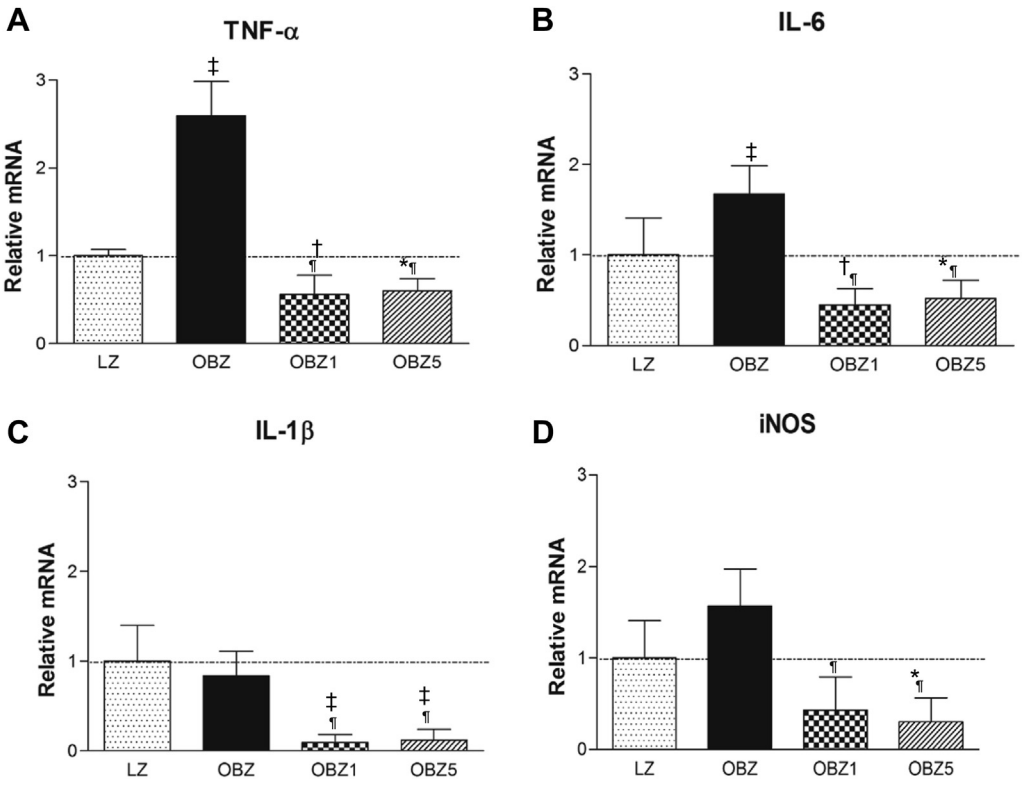

Fig. 3. RBEE effect on gene expression of TNF- (A), IL-6 (B), IL-1 (C) and iNOS (D) in visceral abdominal adipose tissue (VAT) from lean (LZ) and obese Zucker (OBZ) rats determined using real-time polymerase chain reaction of different inflammatory factors. Experimental groups: LZ, lean control; OBZ, obese control; OBZ1, obese rats fed 1% RBEE-supplemented diet; OBZ5, obese rats fed 5% RBEE-supplemented diet. Data are representative of six to seven rats/treatment group and are expressed as mean ± SEM. $^{*}P < 0.05$; $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.001$ represent a significant differences respect LZ sample using one-way ANOVA with a Tukey's Multiple Comparison Test. $^{\P}P < 0.001$ of OBZ1 or OBZ5 with respect to OBZ.

functional abnormalities in adipocytes by attenuating the expression of inflammatory adipokinesin 3 T3-L1 [23]. Previous results have described that RBEE exerts antioxidant [16] and hypocholesterolemic effects [17]. Interestingly, besides tocotrienols, the main bioactive compound of RBEE is γ-oryzanol (1260 mg/kg), which has been suggested to possess lipid-lowering [24], anti-inflammatory [25], anticancer [26], and antioxidant effects [27]. In vivo and in vitro studies have reported that rice bran γ-oryzanol induces anti-inflammatory effects by down-regulating the inflammatory transcription factor, nuclear factor-κB (NF-κB), which in turn dicreases the expression of inflammatory enzymes such as COX-2 and iNOS, and proinflammatory cytokines such as IL-1 β, IL-6, and TNF-α [28]. Accordingly, our results show that an RBEE-supplemented diet clearly improved the inflammation state characteristic of visceral fat in Zucker rats, attenuating the increase of proinflammatory factors such as TNF-α, IL-6, and iNOS, with this effect being more evident in VAT than in VET.

According to previous works describing regional heterogeneity in the mRNA expression of proinflammatory and anti-inflammatory cytokines between different white adipose tissue depots [29], our results show a difference between VAT and VET in the inflammatory feature. The major discrepancy accounts for TNF-α, a cytokine that is strongly linked to obesity and was not increased in VET. It is recognized that TNF-α is more abundantly produced by stromal-vascular cells (mainly macrophages) than by adipocytes [3,5,7]. In this regard, depot differences in cell populations may contribute to depot differences in adipokine production, and can contribute to variations in adipocyte function via paracrine interactions. Interestingly, this study indicates that the effect of RBEE was more evident on the expression of proinflammation factors in abdominal visceral adipose tissue (VAT) than in epididymal fat (VET) on OBZ rats. Further studies are needed to clarify whether the difference in the macrophage infiltration rate into different adipose depots accounts for the difference we have found in the mRNA expression of proinflammatory cytokines. In this line, it is worth noting that macrophages have been identified as the primary source of many of the circulating inflammatory molecules that are detected in the obese state [30] and macrophage infiltration into visceral adipose tissue is higher than into subcutaneous adipose tissues [31], being the expression of proinflammatory cytokines generally higher in visceral than in subcutaneous fat [3, 4].

IL-6 is a proinflammatory cytokine produced by several cell types (fibroblasts, endothelial cells, monocytes, adipocytes), which also are linked to obesity. Thus, the production and circulating level of IL-6 in the obese adipose tissue is increased [3,6]. It is extremely clear from our results, that RBEE has a profound effect on the production of both cytokines in obese rats, particularly in VAT, and there are no major variations on the effects of different RBEE-supplemented diets (1% and 5%).

Endotoxins or inflammatory cytokines, such as TNF-α, normally induce the expression of the iNOS in macrophages [32]. Many inflammatory diseases are accompanied by an increase in NO production and, in appropriate animal models, a beneficial action of iNOS inhibitors has been demonstrated [33]. Interestingly, we found that RBEE-supplemented diets showed a positive

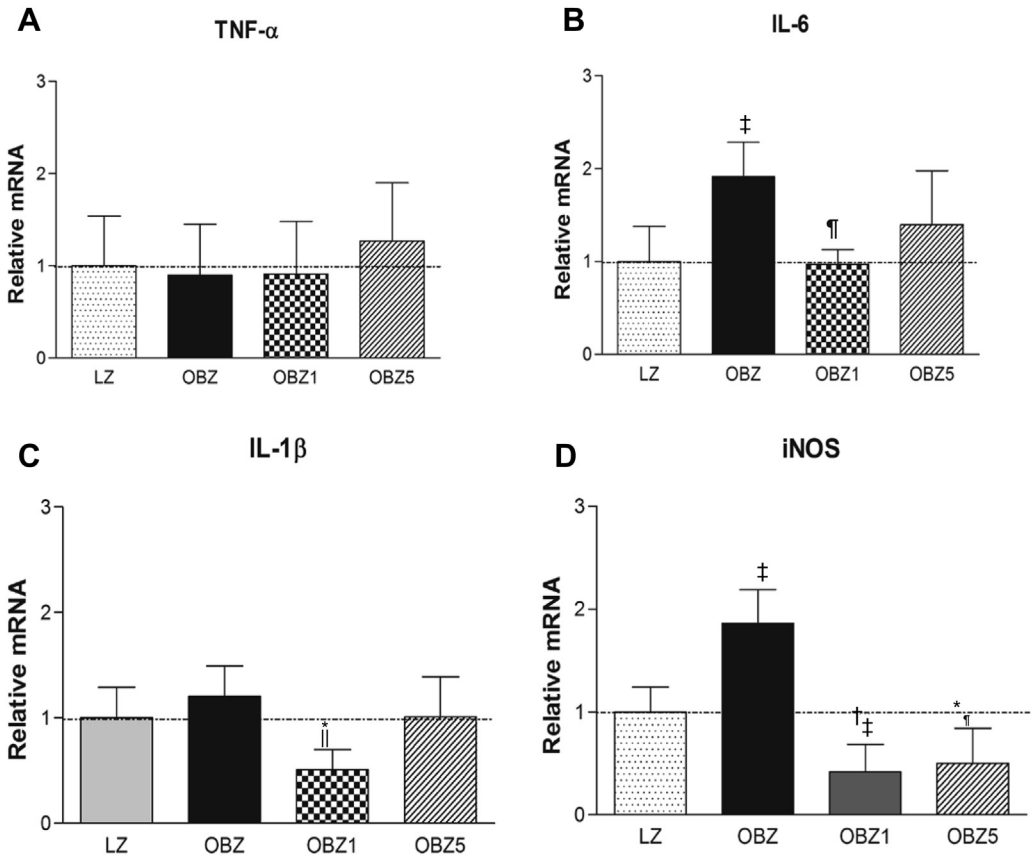

Fig. 4. RBEE effect on gene expression of TNF- (A), IL-6 (B), IL-1 (C) and iNOS (D) in visceral epididymal adipose tissue (VET), from lean (LZ) and obese Zucker (OBZ) rats determined by real-time polymerase chain reaction of different inflammatory factors. Experimental groups: LZ, lean control; OBZ, obese control; OBZ1, obese rats fed 1% RBEE-supplemented diet; OBZ5, obese rats fed 5% RBEE-supplemented diet. Data are representative of six to seven rats/treatment group and are expressed as mean ± SEM. $^{*}P < 0.05$; $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.001$ indicates a significant differences respect LZ sample using one-way ANOVA with a Tukey's Multiple Comparison Test. $^{\P}P < 0.001$ versus OBZ.

effect inhibiting iNOS expression. As stated previously, in obesity there is a remarkable shift in the pool of tissue macrophages from the alternatively activated M2 type to the classically activated M1 type [9]. M2 macrophages are characterized, among other features, by the expression of arginase, an enzyme that blocks iNOS activity, whereas M1 macrophages express not only high levels of proinflammatory cytokines (e.g., TNF-α, IL-6), but also iNOS [34].

Present results show that RBEE-enriched diets did not exert significant results on body weight and fat weight (except in VET). Probably the beneficial effect of RBEE on the inflammatory state in the adipose tissue is mainly carried out on activated macrophage-derived cytokines, more likely than in adipocytes, especially in VAT, in which the anti-inflammatory effect of RBEE was higher. However, we must also consider that the beneficial effect of RBEE on the inflammatory state also may be due, at least partially, to the effect on adipocyte size. Present results show that RBEE induces a general decrease in the adipocyte volume, changing the adipocyte size distribution in rats fed RBEE-supplemented diets in comparison with obese rats fed a standard diet. It has been suggested

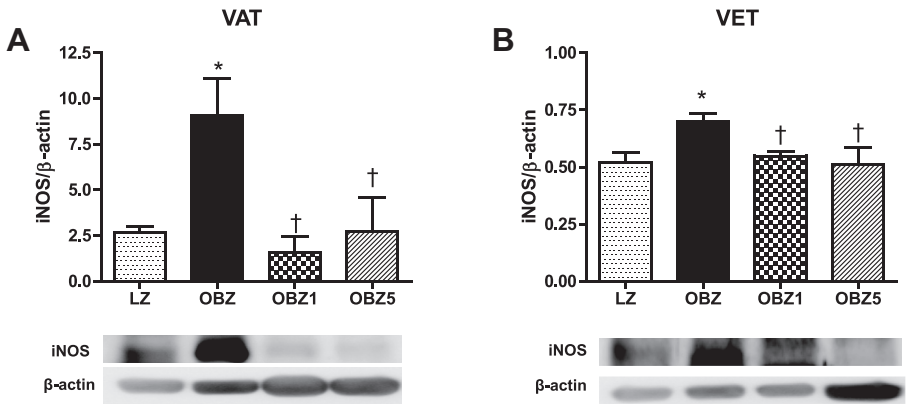

Fig. 5. Expression of iNOS protein in visceral abdominal (VAT) (A) and epididymal tissue (VET) (B) from Zucker rats. Results are the mean ± SEM (n = 4). LZ, lean Zucker control; OBZ, obese Zucker control; OBZ1, 1% RBEE-treated OBZ rats; OBZ5, 5% RBEE-treated OBZ rats. $^{*}P < 0.05$ versus LZ. $^{\dagger}P < 0.05$ versus OBZ.

that in OBZ obese rats, the enlargement of white adipose depots is due to the hypertrophy of adipocytes [35]. Adipocytes enlarge as a consequence of hyperalimentation and large adipocytes release more (saturated) FFAs, which can bind to macrophage toll-like receptor-4 resulting in NF-kB activation, and ultimately leading to augmented TNF-α production [36,37]. In turn, macrophage-derived TNF-α activates human adipocytes, inducing further lipolysis and enhancing the expression of various genes that facilitate the diapedesis of monocytes and consequent differentiation into macrophages [38,39]. Thus, it has been proposed that this local paracrine loop involving adipocyte-derived FFAs and macrophage-derived TNF-α establishes a gradual vicious cycle that presumably leads to a proinflammatory state of both macrophages and adipocytes [21].

Conclusions

In summary, our present work demonstrates that chronic administration of a novel water-soluble RBEE could be a suitable treatment to ameliorate the obesity-associated proinflammatory response. Our results provide evidence of the nutraceutical properties of RBEE against the pathogenesis of obesity and reinforce the potential of RBEE as a functional food.

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ARTÍCULO 5: **Adipose tissue pro-inflammatory response and macrophages polarization associated to obesity is attenuated by a rice bran-supplemented diet in DIO mice**

(Pending submission)

La respuesta pro-inflamatoria del tejido adiposo y la polarización de macrófagos asociada a la obesidad es atenuada por una dieta suplementada con salvado de arroz en ratones DIO.

Justo et al., (Pending submission)

Como se ha indicado anteriormente, la obesidad que participa del proceso patológico que se da en el síndrome metabólico lleva asociada un componente inflamatorio, el cual se origina principalmente en el tejido adiposo como consecuencia de los desequilibrios a nivel estructural y funcional que tienen lugar en los adipocitos [85]. Además, se ha visto cómo en animales obesos tiene lugar un viraje fenotípico en los macrófagos, con tendencia al tipo M1, y de forma paralela, el hígado se ve afectado por esta inflamación sistémica [20, 86]. Habiendo estudiado previamente los efectos de nuestro extracto en un modelo genético de obesidad, nos propusimos como objetivo del presente estudio determinar si un suplemento de RBEE al 1% y 5% podía introducir mejoras a nivel metabólico e inflamatorio en un modelo animal con obesidad inducida por la dieta.

Para ello, durante 16 semanas se les administró una dieta alta en grasa enriquecida al 1% y 5% de EESA a ratones C57BL/6J, estableciéndose dos grupos de tratamiento (HF1% y HF5%), con sus respectivos grupos control, uno de ellos alimentado con dieta alta en grasa (*high fat*, HF) y otro con dieta estándar (*standard*, ST). Una semana antes de finalizar el tratamiento se realizaron tests de tolerancia a la glucosa y resistencia a la insulina. Al finalizar el tratamiento, se determinaron parámetros bioquímicos en suero, y se realizaron cortes histológicos de grasa epididimal para el análisis del tamaño y distribución de los adipocitos mediante la tinción de hematoxilina-eosina, además de la valoración de la presencia de macrófagos por inmunohistoquímica. Por otro lado, se realizó la extracción de material genético de las muestras de grasa e hígado con el fin de determinar los efectos de la dieta en los marcadores más involucrados en el proceso inflamatorio en ambos tejidos, y en la polarización de macrófagos en tejido adiposo.

Esta determinación de la expresión génica se realizó mediante la técnica de la PCR a tiempo real.

Como resultado, se observó que la dieta suplementada con EESA no modificó de forma significativa el peso de los animales obesos en comparación con los animales ST. En cambio, ambas concentraciones de EESA (1% y 5%) atenuaron significativamente los niveles de TG, insulina y nitritos en suero. En el caso del colesterol total, la glucosa y la adiponectina sólo en el grupo HF1% se observó una disminución significativa frente al grupo control obeso. Los niveles de HDL-c no sufrieron cambios con el tratamiento. Respecto a la tolerancia a la glucosa, en el test no se observaron mejoras de los grupos HF comparados con el grupo ST, pero en el caso de la resistencia a la insulina, 1% EESA indujo mejoras significativas frente a los animales HF. También fue patente la recuperación parcial del tamaño y distribución de adipocitos en los animales HF1% en comparación con los controles obesos. Esta recuperación se mostró más ligera en el caso de los ratones HF5%. El análisis inmunohistoquímico con Mac-2 en las secciones de grasa reveló el mismo perfil que mostraron los grupos de tratamiento según el tamaño y distribución de adipocitos. En relación a la expresión génica, se observó cómo el incremento en la expresión de leptina observado en los grupos obesos frente al grupo delgado no sufrió modificaciones. En cambio, la sobre-expresión observada en los animales HF control de adiponectina, PPAR-γ, TNF-α y Emr1 en las muestras de grasa epididimal, se vio significativamente disminuida por 1% EESA, no así con el 5%. Además, el ratio M1/M2 en la grasa epididimal del grupo HF1% fue significativamente menor que en el grupo HF control. En el caso de la expresión de IL-6 e IL-1β en tejido adiposo, y de TNF-α y Emr1 en el hígado, se produjo una atenuación significativa en ambos grupos HF tratados frente al control obeso, tendiendo los valores al grupo ST.

Como conclusión, este estudio pone de manifiesto que la administración crónica de EESA es capaz de mejorar el perfil lipídico, la resistencia a la insulina, la hiperglicemia e hiperinsulinemia en ratones con obesidad inducida por la dieta, demostrando además

que el extracto puede influir en el remodelaje del tejido adiposo y en sus alteraciones funcionales, modelando la producción de macrófagos y sus citoquinas. Por tanto, una dieta suplementada con EESA podría ser utilizada para disminuir el estado pro-inflamatorio asociado al síndrome metabólico y sus complicaciones, cuando éstos tienen su origen en una alimentación inadecuada.

Adipose tissue pro-inflammatory response and macrophages polarization associated to obesity is attenuated by a rice bran-supplemented diet in DIO mice.

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Abstract The inflammatory process associated to obesity in metabolic syndrome mainly arises from white adipose tissue (WAT) alterations, such as macrophages accumulation or adipokines production deregulation. Our aim was to determine if a rice bran enzymatic extract (RBEE)-supplemented diet would ameliorate metabolic, biochemical and functional adipose tissue and macrophages changes associated to a diet-induced obesity (DIO) in mice.

C57BL/6J mice ages 6 wk were divided into three groups, and daily fed high-fat diet (HF), 1% and 5% RBEE-supplemented high-fat diet (HF1% and HF5%, respectively). Another group of mice was fed standard diet (ST). Measurements of serum cardiometabolic parameters, glucose and insulin tests, adipocytes size and mRNA expression of pro-inflammatory biomarkers from epididymal adipose tissue (EAT) and liver were made after 16 wk of treatment.

RBEE administration significantly decreased insulin resistance in obese mice. Serum TG, TC, glucose, insulin, adiponectin and nitrites from treated mice were partially restored, mainly by 1% RBEE-enriched diet. The incremented adipocytes size observed in HF group was reduced by RBEE treatment, being 1% more effective than 5% RBEE. At the same time, pro-inflammatory biomarkers such as IL-6 and IL-1β were significantly decreased in RBEE-treated mice. Other mediators related to obesity such as adiponectin, PPARγ, TNF-α, Emr1 or M1/M2 biomarkers showed significantly restored mRNA levels in WAT from HF1% compared to HF mice.

As a conclusion, this study showed that RBEE administration could act over insulin resistance, dyslipemia and morphological and functional alterations of adipose tissue in DIO mice. These benefits were accompanied by a modulating effect in adipocytes secretion and macrophages polarization. Therefore, RBEE may be considered an alternative nutritional complement able to strongly favor preventive strategies applied to metabolic syndrome and its complications.

***Keywords*:** Rice bran; γ-oryzanol; Obesity; Inflammation; Adipose tissue; Macrophages polarization; DIO mice

Introduction

Inflammation is considered a constant between pathologies related to metabolic syndrome such as obesity, type II diabetes or cardiovascular alterations. Indeed, it is broadly accepted that obesity-associated inflammation leads to the onset of insulin resistance and type 2 diabetes mellitus, linked to some alterations in the remodeling and exocrine function of white adipose tissue (WAT) [1]. In particular, the idea that adipocytes take part of a static organ or a simple energy-store is out-date, since these cells are able to release adipokines with physiological and pathological functions. These mediators include leptin and adiponectin as well as other substances with a negative influence on this pro-inflammatory state such as TNF-α, IL-6 or IL-1β [2, 3]. Simultaneously, the adipose tissue functionality may be impaired also because of changes in adipocyte size [4, 5].

On the other side, several studies have established that the macrophages accumulation and inflammation-related gene expression in white fat depots and liver are often key precedents for obesity and insulin resistance in rodents and humans [6]. Apart from increasing in numbers, adipose tissue macrophages also suffer phenotypical alterations during the metabolic syndrome process. During these pathologies, macrophages evolve from the alternatively-activated M2 type to the classically activated M1 type, changing the secretion of cytokines from predominantly anti-inflammatory (M2) to pro-inflammatory (M1) [7]. It is known that some mediators such as the peroxisome proliferator activator receptor gamma (PPAR-y) play a key role in this macrophages polarization, hence in insulin sensitivity and the regulation of different inflammatory adipokines releasing by adipose tissue, liver and skeletal muscle [8, 9]. Due to the importance of PPARs metabolic functions, it has been described a lot of natural and synthetic PPAR-y agonists like some polyunsaturated fatty acids or glitazones, respectively [10].

Recently, various food derivatives or natural compounds isolated from edible plants that show anti-obesity effect have been screened for its use as functional foods or dietary supplements [11]. Rice bran is an excellent nutritional source of bioactive compounds, including nutraceutical proteins and phytochemicals [12, 13]. Besides, phenolic compounds contained in rice bran, such as γ-oryzanol and ferulic acid, are known to provide strong antioxidant, hypolipidemic and anti-inflammatory activities [14, 15]. The therapeutic use of rice bran is limited because of the insolubility of its proteins, the integrity of its nutraceutical compounds, and the rancidity of its oil-derived. These limitations have been counteracted by the production of the water-soluble rice bran enzymatic extract (RBEE) used in this study [16], that provides numerous advantages over other rice bran derivatives [17-19].

In the last few years, our group has demonstrated the benefits that the novel water-soluble rice bran extract could offer to obese animals through different studies, where a RBEE-supplemented

diet restored alterations associated to metabolic syndrome in Zucker rats such as hypertension, dyslipidemia, vascular alterations, oxidative stress and inflammation derived from adipose tissue [20-23]. However, many questions remained unknown about the mechanisms involved in these beneficial effects, particularly in non-genetic animal models of disease.

As previously described, we have found that the administration of RBEE provides some preventive effects on the complications related to metabolic syndrome, including the homeostasis of some pro-inflammatory biomarkers in a genetic model of obesity. Therefore, the aim of this study was to investigate for the first time if RBEE, administered to another animal model like mice fed a high fat diet, is able to improve the adipose tissue dysfunction, acting as a modulator of the macrophages population changes and the mediators secretion that take place in adipocytes. If the RBEE intake may restore most of the mechanisms that trigger the inflammation process associated to a diet-induced obesity, we could suggest the potential therapeutic use of RBEE as a functional food.

Materials and methods

Animals and diets.-

Male C57BL/6J mice (6 weeks aged, Charles River Laboratories, Barcelona, Spain) were maintained at a temperature and humidity of 21-25°C and 50-60%, respectively, and they were kept on a 12 h light-12 h dark cycle with free access to food and water. After two weeks of adaptation, mice were divided in 2 groups, fed standard diet (ST, Ref. D12450B, *Research Diets Inc.,* NJ, EEUU) and high fat diet (HF, Ref. D12492, *Research Diets Inc.,* NJ, EEUU). Respectively, each group was fed 1% and 5% RBEE supplemented-diet establishing the following groups of treatment for 16 weeks: ST, HF, HF1% and HF5% (n=10 per group).

The extract was administered as syrup mixed with the basal diet according to the group of treatment. RBEE was supplied by the Enzymatic Production Technology Group of the Department of Biochemistry and Molecular Biology, School of Pharmacy, University of Seville (Spain), and was prepared according to Parrado et al. [16]. Chemical composition of RBEE has been previously characterized [16]. Briefly, protein is the major component (38 %) in the form of peptides and free aminoacids due to the use of proteases for rice bran stabilization. The fat components present in RBEE (30 %) are mainly soluble because of protein interactions. Minor functional components of lipid fraction in RBEE include phytosterols (4,084 mg/kg), γ-oryzanol (1,260 mg/kg), tocopherols (99 mg/kg) and tocotrienols (174 mg/kg).

Body weight, food and water intake were weekly evaluated. At the end of treatment, animals were kept fasting for 12h and were anesthetized with chloral hydrate 4% intraperitoneally.

Animals were then sacrificed, and white and brown adipose tissue (abdominal, epididymal and brown fat) and liver were removed and weighted. The protocol for animal handling and experimentation agreed with the European Union European Community guidelines for the ethical treatment of animals (UE Directive of 2010; 2010/63/UE) and was approved by the Ethical Committee for Animal Research of the University of Seville (RD 53/2013).

Biochemical parameters.-

Serum samples were obtained from blood by centrifugation for 20 min at 4,000 rpm and room temperature. Fasting glucose, total- and HDL-cholesterol were assessed by UV/ visible spectrophotometry kits (Spin React, CIMA Diagnostics, Girona, Spain). TG levels were also determined by commercial kits (WAKO Diagnostics, Richmond, VA, USA). Plasma adiponectin and insulin were measured by commercial enzyme-linked immunosorbent assay (ELISA) kits (B-Bridge International, Otsuka, Japan; Millipore, Missouri, USA). The homeostasis model assessment of insulin resistance and glucose tolerance was assayed as previously described [24]. Serum levels of nitric oxide metabolites (NO(x)) were determined by using nitrate reductase to specifically reduce nitrate to nitrite; the latter was quantified by a colorimetric assay using the Griess reagent [25].

Glucose tolerance and insulin resistance tests.-

At 14 weeks of treatment, mice were subjected to oral glucose tolerance and insulin resistance tests. The oral glucose tolerance test was performed by oral administration of glucose (2 g/kg body weight) to experimental groups previously fasted for 14 h. Blood samples were obtained from the tail vein at the assay starting in order to determine basal levels of glucose in plasma, and after 30, 90 and 120 min of glucose administration. Plasma glucose concentration was determined using a blood glucose commercial monitoring meter (Accutrend®Plus_GCTL; Roche Diagnostics, Barcelona, Spain). For insulin resistance test, food was withdrawn 3 h before the test and mice were injected intraperitoneally with insulin (100 IU/mL; Humulina Regular, Lilly S.A., Spain). Blood samples were collected at the same time intervals. The area under the glucose curve from both tests was calculated using Prism GraphPad 5.01 software (San Diego, CA, USA). For glucose tolerance and insulin resistance data, each value is the total area under glucose curve for each group of treatment. To simplify the representation of glucose changes from baseline during the test, percentages are shown graphically.

Measurement of adipocyte size.-

Epididymal adipose tissue (EAT) from mice was fixed in 7,5 % paraformaldehyde and embedded in paraffin blocks using conventional histological techniques and sectioned at 4 μm. Hematoxylin- and eosin-stained slides from adipose tissue were analyzed. Four different representative microscopic fields were captured manually from sections of each animal (five

animals per group) and measured by using ImageJ 1.42 software (Bethesda, Maryland, USA). To determine average of area of adipocytes and size distribution was traced manually and measured in 100 cells per mouse for each experimental group at 10x magnification (Olympus, Hamburg, Germany) [23].

Immunohistochemistry .-

For immunohistochemistry 4 μm dewaxed serial sections were incubated with anti-Mac-2 (Cedarlane Laboratories, Canada) on adipose tissue using the ABC kit (Vector Laboratories; Burlingame, CA, USA) according to the manufacturer´s recommendations. As a negative control, staining was performed on selected sections with isotype control. Biotinylated HRP-conjugated secondary antibodies was horse anti-mouse IgG (Vector Laboratories; Burlingame, CA, USA). Samples were captured at 20x magnification by light microscopy (Olympus, Hamburg, Germany).

RNA isolation and qRT-PCR.-

Parts of EAT and liver were immediately snap frozen in liquid nitrogen for RNA isolation. Adipose tissue and liver was homogenized in Trizol reagent (Invitrogen), and RNA was isolated according to manufacturer's protocol. One microgram of total RNA was treated with DNase I and reverse transcribed into cDNA using Superscript II and random hexamer primers (all Invitrogen). Gene expression normalized to Ubiquitin C was analyzed by quantitative real-time RT-PCR on an ABI Prism 7000 cycler using commercial assays-ondemand kits (all Applied Biosystems, Foster City, CA).

Statistical analysis.-

Data represented are mean ± SEM of n = 10 mice. One-way ANOVA with Dunnet´s post-hoc comparison was used to compare data. Differences were considered significant when $P < 0.05$. A Prism GraphPad 5.01 software (San Diego, CA, USA) was used for statistical analysis.

Results

Body weight, food intake and organ weights.-

Final body weight in animals fed HF diet was significantly higher ($P<0.001$) than in the animals fed ST diet during the treatment (Table 1). Despite the variability and no significant differences in caloric and food intake values, significant differences were appreciated in final body weight (Table 1) and body weight gain (Fig. 1) between control and obese animals. Nevertheless, body weights were not significantly modified by RBEE administration (Table 1 and Fig. 1).

Regarding to the adipose tissue, no differences were observed between the groups of treatment in brown fat weights. However, weight of white adipose tissue was significantly higher in HF, HF1% and HF5% groups (P<0.001) than in ST animals. Liver weight from HF and HF1% mice was slightly higher (P<0.05) than that from ST and HF5% mice (Table 1).

Table 1.- Food and caloric intake, final body and relative organ weights of the different groups of treatment at the end of the study. Mean ± SEM (n=10)

		ST	HF	HF1%	HF5%
Food intake (g/wk/animal)		22.62 ± 5.65	30.88 ± 10.92	17.05 ± 4.73	20.77 ± 8.48
Caloric intake (kcal)		87.08 ± 21.77	161.80 ± 57.20	89.27 ± 24.76	108.39 ± 44.25
Final body weight (g)		28.48 ± 0.39	41.70 ± 1.51 $^{###}$	37.63 ± 1.86 $^{###}$	40.10 ± 1.59 $^{###}$
Organ weights (g/100g body weight)					
Adipose Tissue (AT)	**Abdominal AT**	0.58 ± 0.08	2.21 ± 0.17 $^{###}$	1.61 ± 0.10 $^{### *}$	2.19 ± 0.14 $^{###}$
	Epididymal AT	2.02 ± 0.18	5.99 ± 0.32 $^{###}$	5.08 ± 0.47 $^{###}$	5.92 ± 0.50 $^{###}$
	Brown AT	0.21 ± 0.01	0.24 ± 0.02	0.16 ± 0.01	0.29 ± 0.03
Liver		3.69 ± 0.08	3.11 ± 0.14 $^{#}$	3.07 ± 0.12 $^{#}$	3.42 ± 0.20

Experimental groups: ST, mice fed standard diet; HF, mice fed high fat diet; HF1%, mice fed 1% RBEE-supplemented high fat diet; HF5%, mice fed 5% RBEE-supplemented high fat diet. Data are mean ± SEM (n=10). $^{#}P < 0.05$, $^{###}P < 0.001$ *vs* ST; $^{*}P < 0.05$ *vs* HF.

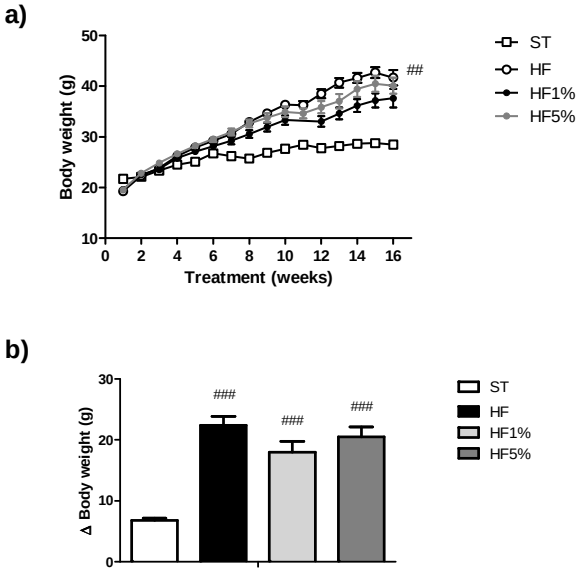

Fig. 1 Body weight evolution (a) and body weight gain (b) of DIO mice during 16 weeks of treatment. ST, mice fed standard diet; HF, mice fed high fat diet; HF1%, mice fed 1% RBEE-supplemented high fat diet; HF5%, mice fed 5% RBEE-supplemented high fat diet. Data are mean ± SEM (n=10). $^{##}P < 0.01$, $^{###}P < 0.001$ *vs* ST.

Biochemical parameters.-

Serum analysis showed levels of TC significantly higher in HF (P<0.01) and HF5% (P<0.001) than in ST; while the concentration of TC in HF1% mice was reduced compared to HF control group (P<0.05), HF5% mice experimented an increase in serum TC (P<0.05) in comparison with HF mice levels. No changes were observed between ST and HF mice in HDL-c and TG levels, but the treatment was able to significantly decrease serum TG in HF mice (P<0.001).
In the case of insulin and glucose levels, HF mice evidenced higher concentrations than ST mice (P<0.001). 1% RBEE attenuated glucose (P<0.05) and insulin (P<0.01) levels, whereas 5% RBEE was only able to decrease insulin levels in mice fed HF diet (P<0.05).
Regarding to other biomarkers tested, 1% RBEE treatment significantly increased serum adiponectin levels in HF mice (P<0.01), and both concentrations of RBEE reduced nitrites in serum compared to HF control mice (1% RBEE, P<0.01; 5% RBEE, P<0.001) in a concentration-dependent manner.

Table 2.- Biochemical analysis after chronic treatment with RBEE. Mean ± SEM (n = 10)

	ST	HF	HF1%	HF5%
TC (mmol/L)	3.16 ± 0.09	3.65 ± 0.13 $^{\#\#}$	3.07 ± 0.19 *	4.27 ± 0.19 $^{\#\#\#\,*}$
HDL-c (mmol/L)	0.93 ± 0.11	1.24 ± 0.10	0.88 ± 0.13	1.31 ± 0.13
TG (mmol/L)	0.53 ± 0.06	0.54 ± 0.05	0.27 ± 0.004 ***	0.27 ± 0.03 ***
Glucose (mmol/L)	4.72 ± 0.58	10.55 ± 0.99 $^{\#\#\#}$	7.80 ± 0.40 $^{\#\,*}$	10.19 ± 0.46 $^{\#\#\#}$
Insulin (ng/mL)	2.23 ± 0.01	2.74 ± 0.17 $^{\#\#\#}$	2.36 ± 0.05 **	2.46 ± 0.04 $^{\#\,*}$
Adiponectin (ng/mL)	6.15 ± 0.76	0.70 ± 0.05 $^{\#\#\#}$	1.32 ± 0.09 $^{\#\#\#\,**}$	1.01 ± 0.11 $^{\#\#\#}$
NO(x) (µmol/L)	7.44 ± 0.26	10.62 ± 1.05 $^{\#\#}$	7.59 ± 0.27 **	4.13 ± 0.25 $^{\#\#\,***}$

ST, mice fed standard diet; HF, mice fed high fat diet; HF1%, mice fed 1% RBEE-supplemented high fat diet; HF5%, mice fed 5% RBEE-supplemented high fat diet. TC: total cholesterol, HDL-c: high-density lipoproteincholesterol, TG: triglycerides. Data are mean ± SEM (n=10). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* ST; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* HF.

Glucose tolerance and insulin resistance tests.-

Plasma glucose concentrations obtained in the oral glucose tolerance test at 15 weeks of treatment revealed significant differences between ST and HF control animals (P<0.001) (Fig.2a). In addition, the analysis of the area under the plasma glucose curve confirmed that HF presented an altered glucose tolerance compared to ST mice (Fig. 2c). Treatment with RBEE did not modify this condition in treated-HF mice versus the HF control group (Fig. 2c).

Insulin resistance test showed a marked alteration in the insulin function after a charge of glucose, mainly in HF compared to ST mice (P<0.05) (Fig. 2b). 1% RBEE was able to improve in

a significant manner the profile (P<0.01), as it is shown by the area under glucose concentration curve (Fig. 2d). However, mice fed 5% RBEE seem to tend to an amelioration of insulin functionality, but these changes were not significantly different to those observed in HF mice (Fig. 2d).

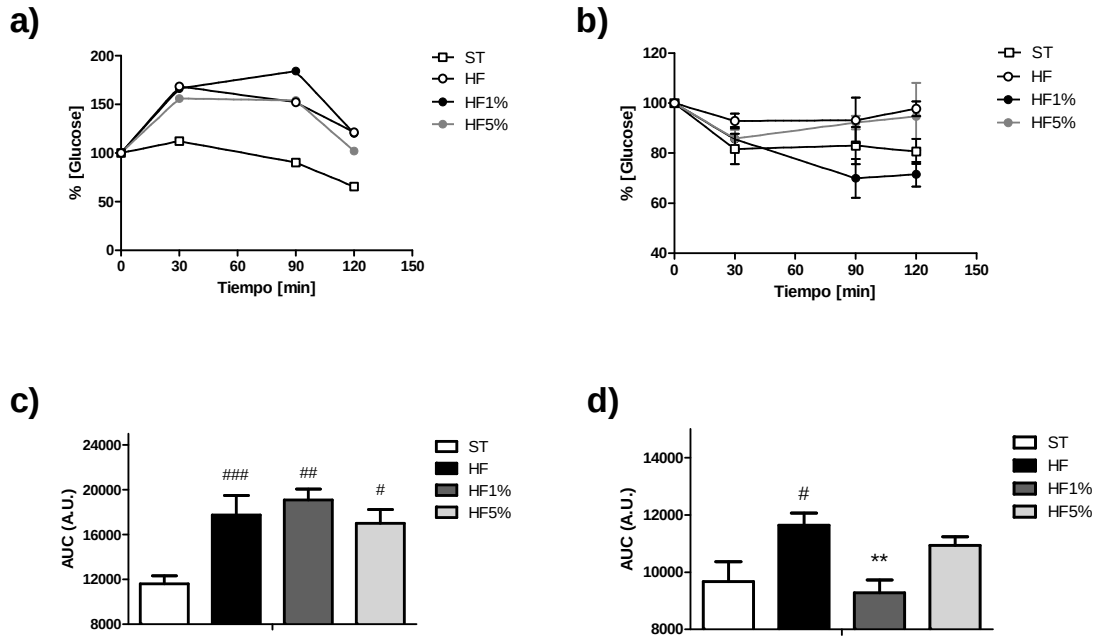

Fig. 2 Physiological tests related to glucose homeostasis. Profile of serum glucose changes obtained from oral glucose tolerance (a) and insulin resistance tests (b) at 15 weeks of treatment. Area under curve (AUC) that results of serum glucose concentrations in the glucose tolerance test (c) and insulin resistance test (d). ST, mice fed standard diet; HF, mice fed high fat diet; HF1%, mice fed 1% RBEE-supplemented high fat diet; HF5%, mice fed 5% RBEE-supplemented high fat diet. Data are mean ± SEM (n = 10). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* ST; $^{**}P < 0.01$ *vs* HF.

Adipocytes size distribution.-

Sectioned EAT showed morphological differences between ST and HF control groups (Fig.3a), where hematoxylin-eosin staining evidenced that only samples from 1% RBEE treated animals had similarities with samples from ST group (Fig.3a).

After quantification, we confirmed that HF control group had significantly larger cells that ST group. Indeed, whole set of cells that have been quantified from ST mice showed an area < 100 μm^2, while adipocytes from HF mice had the main ranges of size distribution included in 100-300 μm^2 cells. About RBEE-treated animals, most of cells in both HF1% and HF5% groups had a major distribution area ranging from 0-200 μm^2, while most of cells from HF control mice showed an area close to 200 μm^2 (Fig. 3b). The most important differences between HF control and RBEE-treated animals were in the range of adipocytes size distribution < 100 μm^2, since 1% RBEE restored the adipocytes size in a 25% of frequency, and 5% RBEE induced an amelioration

of 7% of frequency on this range of distribution. In summary, 1% RBEE-supplemented diet seems to induce a marked shift in adipocytes size distribution in HF mice towards ST mice distribution profile (Fig. 3d). This improvement results from an increment in smaller cells versus HF control mice adipose tissue. In this case, 5% RBEE treatment seems to have a similar but slighter effect than HF1%.

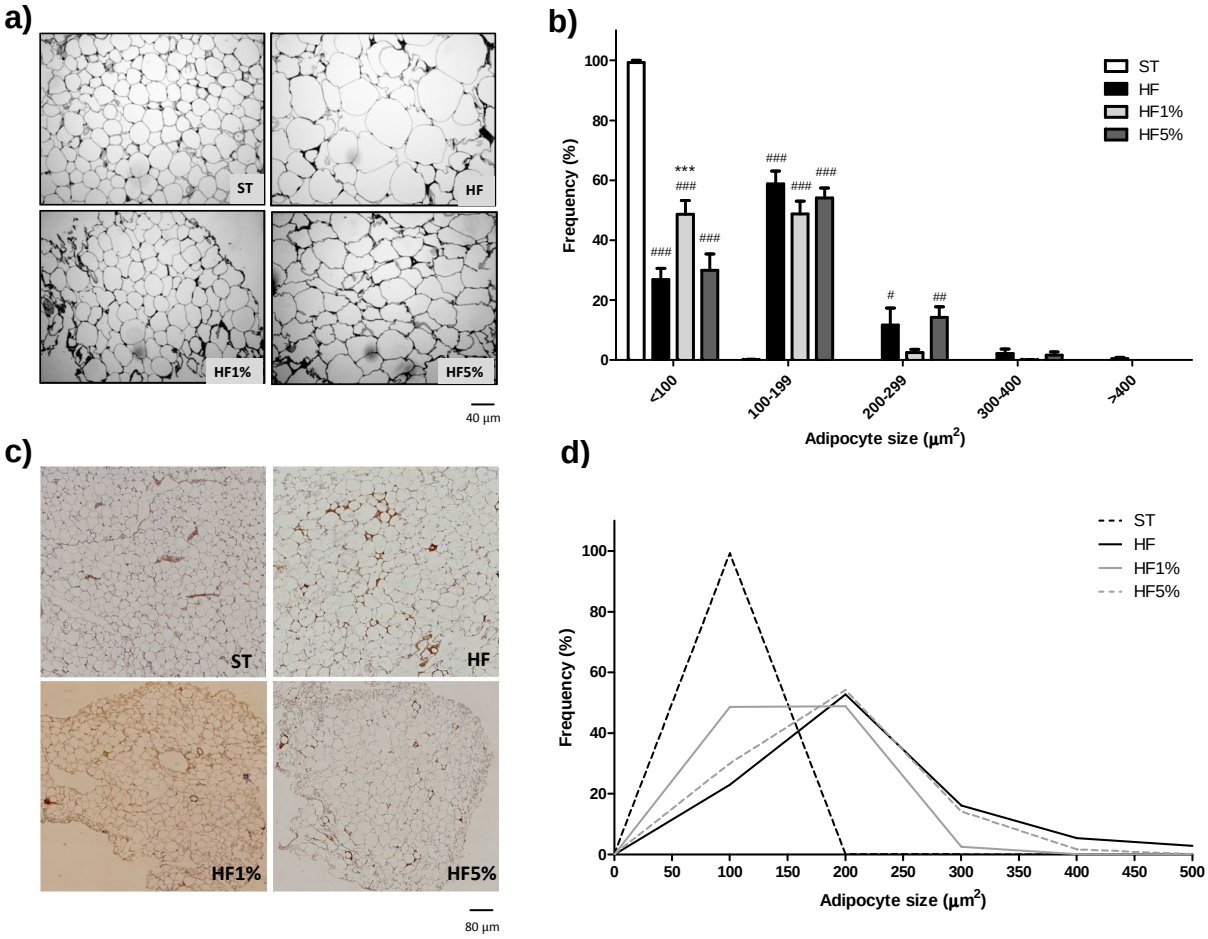

Fig. 3 Histology of epididymal white adipose tissue (EAT) (a) and immunohistochemical staining of Mac-2$^+$ (c). Graphs show distribution (frecuency expressed as %) of adipocyte size (µm) in each experimental group (b, d) . ST, mice fed standard diet; HF, mice fed high fat diet; HF1%, mice fed 1% RBEE-supplemented high fat diet; HF5%, mice fed 5% RBEE-supplemented high fat diet. Data are mean ± SEM (n=10). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* ST; $^{***}P < 0.001$ *vs* HF.

Immunohistochemistry.-

Adipose tissue macrophages accumulation was determined by immunohistochemical staining of Mac-2$^+$ in WAT samples isolated from mice. Representative pictures of every group of treatment showed an incremented presence of macrophages crowns-like structures in obese control HF mice adipose tissue compared to ST group (Fig. 3c). In the case of RBEE-treated animals, it could be appreciated a decreased number of macrophages in samples from both HF1% and HF5% groups, being this effect more tangible in HF1% mice (Fig. 3c).

mRNA expression of biomarkers in adipose tissue.-

An increment of leptin expression was observed in treated and non-treated HF mice compared to ST mice (P<0.001) while there were no significant changes between the obese mice in the mRNA expression of this hormone (Fig. 4a). Adiponectin expression was significantly decreased in HF control mice versus ST group (P<0.01) and this expression was significantly restored by 1% RBEE (P<0.05) whereas remained unchanged in 5% RBEE-treated mice (P<0.01) (Fig. 4b). In the case of PPAR-γ expression, we found a reduced expression level in HF control mice (P<0.001) and 5% RBEE-treated mice (P<0.01) compared to ST animals. In contrast, 1% RBEE-supplemented diet induced an up-regulation of PPAR-γ expression over 30% more than HF group (P<0.001) (Fig. 4c).

With a similar profile, the increment observed in TNF-α expression in HF control (P<0.001) and 5% HF mice (P<0.01) compared to ST animals was significantly attenuated by 1% RBEE-supplemented diet (P<0.01) (Fig. 4d). Level of Emr1 expression was significantly augmented in HF mice (P<0.001 *vs* ST) but these levels tend to decrease in RBEE-treated groups, mainly in HF1% mice (P<0.05) (Fig. 4e). Also the incremented expression of IL-6 and IL-1β that showed HF mice compared to ST mice (P<0.05 and P<0.01, respectively), were restored by both concentrations of the extract, 1% and 5% RBEE (P<0.05 and P<0.01, respectively) (Fig. 4f, g).

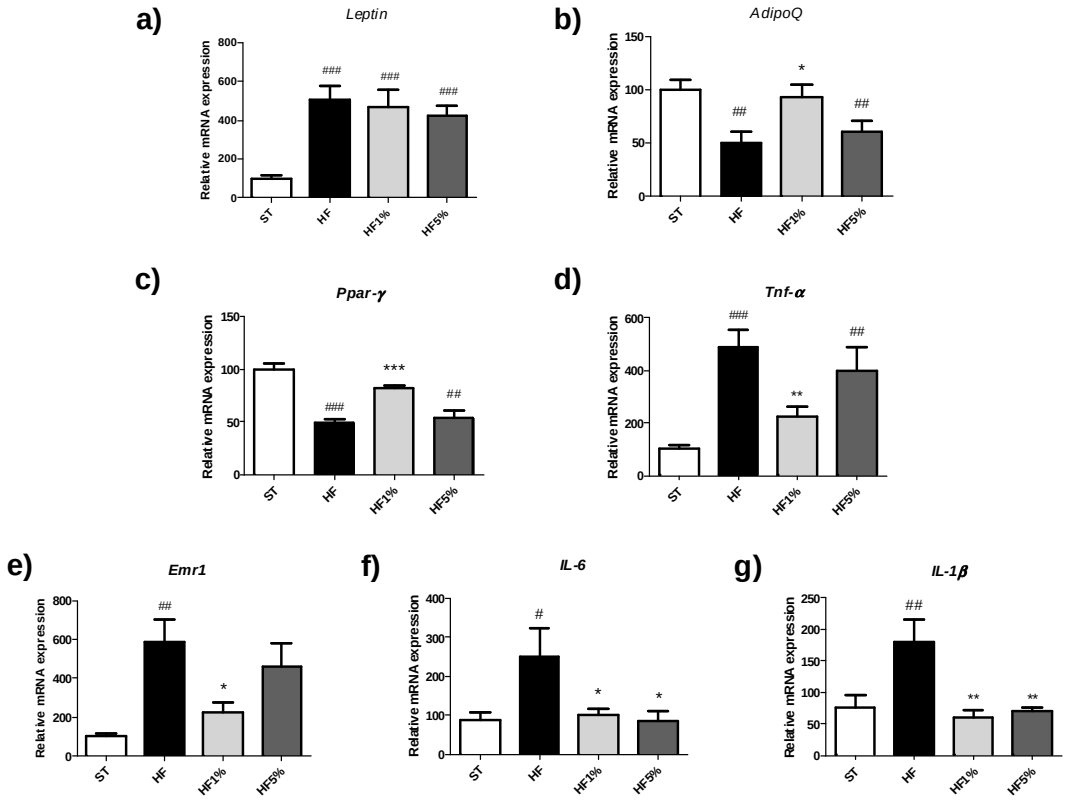

Fig. 4 RBEE effects on gene expression of leptin (a), adiponectin (b), PPARγ (c), TNF-α (d), Emr1 (e), IL-6 (f) and IL-1β (g) in epididymal adipose tissue (EAT) from mice determined by real-time PCR of different inflammatory factors. Data are mean ± SEM (n = 10). ST, mice fed standard diet; HF, mice fed high fat diet; HF1%, mice fed 1% RBEE-supplemented high fat diet; HF5%, mice fed 5% RBEE-supplemented high fat diet. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* ST; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* HF.

Polarization M1/M2 biomarkers in adipose tissue.-

Gene expression of Itgax and MRC1 was significantly augmented in control animals fed HF diet compared to ST mice (P<0.001). The treatment of HF mice with 1% RBEE supplementation was able to reduce mRNA levels of both biomarkers with significant statistical differences (P<0.01 and P<0.001 for Itgax and MRC1, respectively) (Fig. 5a, b). However, these two parameters were not significantly modified by the administration of 5% RBEE (Fig. 5a, b).

Finally, in order to determine if the treatment with RBEE was able to favor the macrophages conversion from M1 to M2 type, we found that the ratio Itgax/MRC1 was significantly increased in HF and 5% RBEE mice (P<0.001) compared to ST, whereas it was notably reduced in 1% RBEE-treated animals (P<0.01) (Fig. 5c).

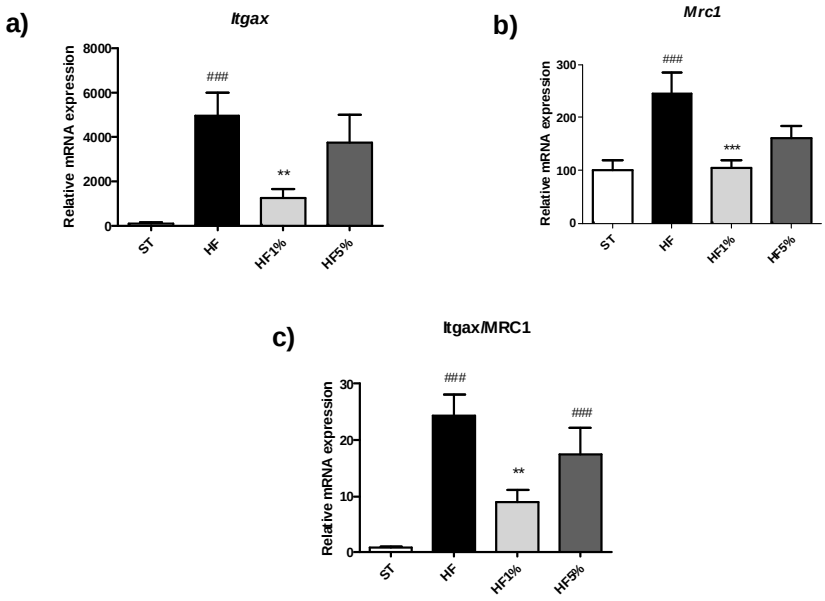

Fig. 5 Changes induced in the gene expression of biomarkers related to M1/M2 macrophages polarization, such as Itgax (a), MRC1 (b) and the ratio Itgax/MRC1 (c). Samples obtained from epididymal adipose tissue (EAT) from mice determined by real-time PCR. Data are mean ± SEM (n = 10). ST, mice fed standard diet; HF, mice fed high fat diet; HF1%, mice fed 1% RBEE-supplemented high fat diet; HF5%, mice fed 5% RBEE-supplemented high fat diet. $^{###}P < 0.001$ *vs* ST; $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* HF.

mRNA expression of biomarkers in liver.-

Analysis of liver samples revealed that the increased gene expression of Emr1 (Fig. 6a) and TNF-α (Fig. 6b) in the HF control group (P<0.05) was normalized by RBEE treatment in both cases (P<0.05). However, PPAR-α mRNA expression remained unchanged in the four experimental groups (Fig. 6c).

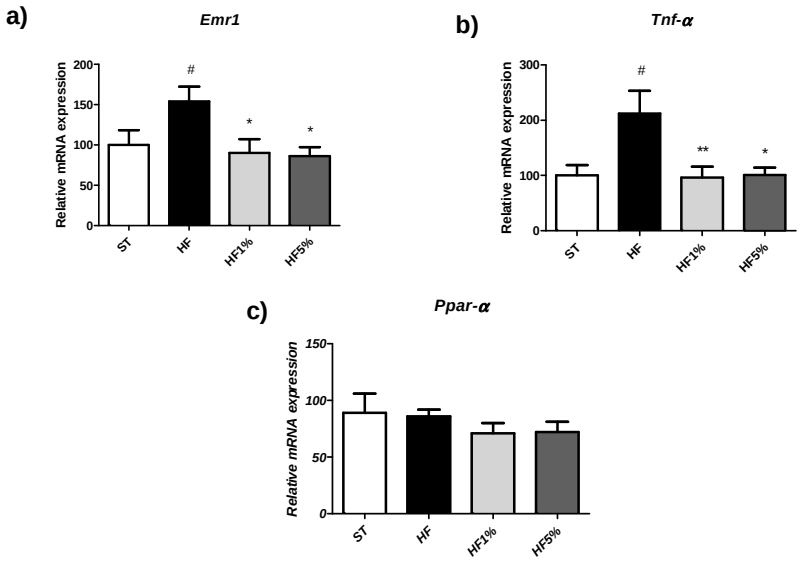

Fig. 6 Effects of the treatment with RBEE-supplemented high fat diet induced in the gene expression of different pro-inflammatory factors in liver. Samples obtained from mice determined by real-time PCR. Data are mean ± SEM (n = 10). ST, mice fed standard diet; HF, mice fed high fat diet; HF1%, mice fed 1% RBEE-supplemented high fat diet; HF5%, mice fed 5% RBEE-supplemented high fat diet. $^{\#}P < 0.05$ *vs* ST; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* HF.

Discussion

The interest of this study was to evaluate if the administration of a RBEE-supplemented diet could provide benefits over some pathological processes related to metabolic syndrome developed in animals with a diet-induced obesity (DIO). In this investigation, we have been focused in the analysis of metabolic and biochemical parameters and specifically we have examined for the first time how the RBEE-enriched high fat diets affect to the morphological and functional alterations in the epididymal adipose tissue (EAT) of obese mice. This study demonstrates for the first time how RBEE supplementation is able to generate beneficial changes not only in the adipose tissue structure, but also in the adipokines production and in the key mediators for macrophages polarization.

Although neither final body weight nor the EAT weight showed significant differences between the different groups of treatment, there were slight differences in food intake, since RBEE-treated groups seem to have eaten less than the HF control group, even approaching to ST group levels (Table 1). The possible explanation could be that γ-oryzanol, the major bioactive component of RBEE, has been described as a substance with a hypothalamic activity which attenuates the preference for dietary fat in the animals [16].

Regarding to the white fat weight, we have only observed differences in visceral adipose tissue, where 1% RBEE treatment reduced this fat mass in mice fed HF diet, in spite of that HF control and 5% RBEE kept higher weights than ST animals. However, the administration of the extract

induced no changes in EAT of the treated and non-treated obese mice (Table 1). This fact agrees with other studies where the supplementation of rice or other nutraceutical products such as rosehip in high fat diets have modified other white fat weights on this animal model, but not epididymal fat weight [11, 27].

About the lipid profile, extracts of rice bran have demonstrated its ability to reduce TC and LDL-c, and to increase HDL-c levels in serum from different experimental models [28-30]. This action has been attributed to the major components that take part in the composition of rice bran, such as unsaturated fatty acids, triterpene alcohols, phytosterols, tocotrienols and tocopherols, and γ-oryzanol (a mixture of phytosteryl ferulates). Ferulic acid is another important compound present in rice bran, and several authors have described its beneficial activity through *in vitro* and *in vivo* assays [31, 32]. These studies show the advantages of a treatment with ferulic acid and its derivatives on oxidative stress, vascular dysfunction, dyslipidemias and inflammation associated to obesity [33, 34]. Previously, the supplementation with ferulic acid to DIO mice showed that this substance reduced TG and TC levels in serum, the extent of lipid peroxidation, down regulated the translocation and expression of NF-κB, cytokine level of TNF-α, IL-6 and restored the endogenous antioxidant status by quenching the free radicals produced by a high fat diet [35]. In the case of RBEE, these properties related with its anti-hyperlipidemic effects gain even more interest because of its enrichment in this last group of molecules derived from ferulic acid that is γ-oryzanol [14, 20].

Obesity and insulin resistance, cardinal features of metabolic syndrome, are closely associated with a state of low-grade inflammation [8, 36, 37]. Although liver and muscle show obesity-induced mild inflammatory responses, white adipose tissue (WAT) is the key site mediating systemic inflammation [38]. With the development of obesity, WAT undergoes a tissue remodeling in which adipocytes increase in both number (hyperplasia) and size (hypertrophy). Metabolic derangements associated with obesity, including type 2 diabetes, occur when WAT growth through hyperplasia and hypertrophy cannot keep the balance between the energy storage needs and the chronic energy excess. Accordingly, hypertrophic adipocytes become overburdened with lipids, resulting in changes in the secreted hormonal milieu [39]. Adipose tissue secretes more than 50 hormones called adipokines, which exert their biological roles in an autocrine, paracrine or systemic manner and influence several physiological processes concerning energy, glucose metabolism, and immunity [40, 41]. Some of these adipokines are involved in the insulin resistance development, such as adiponectin, leptin, TNF-α or IL-6. In cultured murine adipocytes, IL-6 production is strongly increased by TNF-α and induces insulin resistance by inhibiting glucose uptake and impairing insulin signaling and action [42]. In a previous study, RBEE showed its anti-inflammatory action by decreasing mRNA levels of TNF-α, IL-6, IL-1β and iNOS in visceral and epididymal WAT from Zucker rats [23]. Although Candiracci *et al.* did not appreciate important differences in the anti-inflammatory effect of 1% and 5% RBEE administration, only 1% RBEE induced a significant decrease in IL-6 and IL-1β mRNA levels from EAT. In the present investigation we have found that only 1% RBEE

significantly improved parameters directly related to WAT paracrine function and background (adiponectin, TNF-α, PPARγ or Emr) (Fig. 4b, c, d, e).The fact that γ-oryzanol had been studied as an anti-inflammatory and a promotor of adiponectin increasing [43, 44] could be linked to the attenuation of the insulin resistance state that 1 % RBEE treatment produced in DIO mice (Fig. 2b, d). However, characteristic mediators released by macrophages such as IL-6, IL-1β (Fig. 4f, g) or liver inflammation markers (Fig.6a, b) have been similarly attenuated in both HF1% and HF5% mice. This finding agrees with our vascular functionality results, where it is shown that the extract develops a potent antioxidant activity at both 1% and 5% concentrations as well as a significant reduction of TNF-α and iNOS vascular expression [21].These data reflect that RBEE could act in a different manner depending of the ROS involvement, since the extract preserves its effectiveness in both 1% and 5% contribution always that the pathological condition has a basis in the oxidative damage. On the opposite, 1% RBEE seems to be a selective concentration to attenuate ROS-independent risk factors associated to obesity. However, more experiments are in progress to confirm this hypothesis.

Apart from increasing in numbers, the hypertrophy of adipocytes triggers to an infiltration of immune cells, mainly macrophages which are going to acquire an essential role in the releasing of different inflammatory mediators. Adipose tissue macrophages are also phenotypically changed during obesity: while anti-inflammatory M2 macrophages reside in WAT of lean mice, obese WAT predominantly contains M1 macrophages, a prominent source of pro-inflammatory cytokines such as TNF-α and IL-6 [45]. According to our results, 1% RBEE administration decreased Emr1 expression in a significant manner (P<0.05) against of the increased values found in HF control mice (P<0.01) (Fig. 4e). Moreover, immunohistochemical assay on EAT sections shows how macrophages recruitment appears incremented in HF control mice, while RBEE-treated mice have a lowered number of macrophages crowns (Fig. 3d). So, RBEE treatment could have influence in the macrophages polarization towards to M2 population. This fact was confirmed thanks to the determination of the M1/M2 ratio, which was made with genes related to both populations, Itgax/MRC1. In effect, 1% RBEE-supplemented diet significantly reduced this value compared to HF group (Fig. 5). This finding could be a consequence of the increased adiponectin and PPARγ expression that 1% RBEE supplement induced in this experiment (Fig. 4b, c), since they have been described like inductors of the M2 macrophages [46]. Besides, insulin resistance is associated with both the number of M1 macrophages and an elevated M1-to-M2 ratio, condition that also was restored by 1% RBEE (Fig. 2b, d). In relation to this, there are drugs, like pioglitazone, able to improve this ratio and therefore, the insulin resistance state, as observed with RBEE treatment [47].

On the other side, dietary components may up-regulate the expression of the PPARγ as their mechanism of action towards disease prevention [48]. Components like tocopherol, linoleic acid, curcumin and resveratrol upregulate PPARγ expression with no reported side effects in chronic diseases like obesity, cardiovascular diseases, colon cancer and diabetes [49]. It has been demonstrated that compounds of whole rice grain improve metabolic states through an increase

of PPARγ expression, while the down-regulation seems to have also benefits against obesity. Besides, insulin has also been reported to influence the expression of PPARγ [50]. In the presence of insulin, PPARγ expression increases beyond basal levels, and our results also show this effect (Fig. 4c). However, it has been tested that GABA, oryzanol and acylated steryl glycoside (ASG), characteristic components of rice bran, may be more effective than insulin on PPARγ expression, improving metabolic indices in biological systems even in insulin-resistant conditions [51, 52].

Finally, we have observed that the 1% and 5% RBEE treatment induced a significant amelioration of the liver inflammation in contrast to that shown in HF control mice, by reducing Emr1 and TNF-α expression (Fig. 6a, b). However, there were no changes in the PPARα expression. It has been reported that the expression of PPARα in the liver is up-regulated by glucocorticoids, n-3 polyunsaturated fatty acids and fasting, but is down-regulated by insulin [53-55]. Also, it has been demonstrated that obesity-associated hyperleptinemia may have a role in the induction of liver PPARα gene expression. Therefore, we could suppose that if leptin levels have not been modified in this study by the RBEE-treatment, also PPARα expression could have no changes between the treated and non-treated animals fed a high fat diet [56].

As a conclusion, the present study demonstrates that chronic administration of a novel water-soluble RBEE is able to ameliorate lipid profile, insulin resistance, hyperglycemia and hyperinsulinemia in DIO mice. At the same time, the treatment with the extract considerably reduces the pro-inflammatory state linked to metabolic syndrome. This is the first work were RBEE acts as a modulator in the deterioration of adipose tissue morphology, function and macrophages population in the development of metabolic syndrome. Therefore, RBEE could be considered an ideal complement in combination with the preventive and therapeutic strategies against the obesity-associated complications.

Acknowledgments

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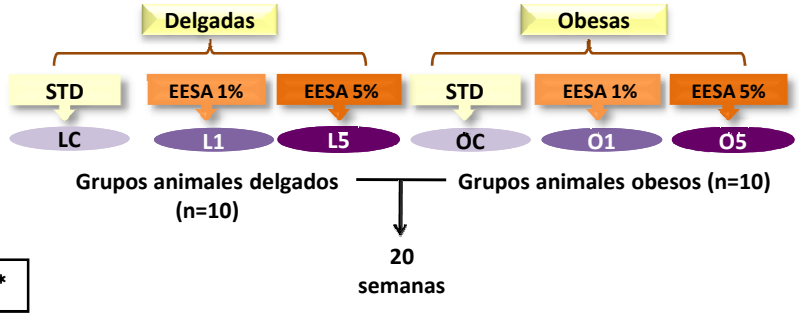
RESUMEN GLOBAL DE LOS RESULTADOS

Resumen global de resultados obtenidos en el modelo genético de ratas Zucker obesas

Objetivos

Estudiar los efectos beneficiosos de una dieta suplementada con un extracto enzimático de salvado de arroz (EESA) en la restauración de las alteraciones asociadas al Síndrome Metabólico que desarrollaron ratas Zucker obesas, a nivel cardiometabólico, vascular e inflamatorio.

Diseño experimental



Resultados *

Efecto cardiometabólico

- Disminución PAS de forma concentración-dependiente
- EESA 5% evitó el desarrollo de resistencia a la insulina
- -Los niveles de CT , HDL-c, TG, insulinemia, adiponectina y nitritos en suero mejoraron de forma concentración-dependiente en los animales obesos tratados.
  - En el caso de EESA 5% se produjeron mejoras del índice aterogénico, HOMA-IR , y TG y CT en hígado.

Efecto vascular

Arterias de conductancia

- Disminución de la disfunción endotelial y de la producción de anión superóxido en la aorta
- Recuperación del papel de NO en la vasorrelajación con EESA 5%, y aumento de la expresión proteica de eNOS por EESA 1% y 5%
- Restauración de la implicación de los derivados dependientes de endotelio en la vasocconstricción gracias a EESA 1% y 5%
- Restauración de los niveles de mRNA tratados con EESA 1% y 5% de iNOS, TNF-α y las subunidades de la NADPH oxidasa NOX-1 y $p22^{phox}$

Arterias de resistencia

- Aumento de la contribucón de los EDHF por EESA 1% y 5% en la vasorrelajación del vaso mesentérico
- Incremento por parte de EESA 5% de la participación de NO en la capacidad vasodilatadora
- Sobre-expresión proteica por parte de EESA 1% y 5% de los SK_{Ca}, y por parte de EESA 5% de los IK_{Ca} y la enzima eNOS
- Disminución de la expresión de iNOS en la pared arterial
- Atenuación de la producción de anión superóxido en la arteria

Efecto en tejido adiposo

Grasa epididimal:
- Notables mejoras en la distribución de tamaño de los adipocitos por parte de EESA 1% y 5%.
- Disminución significativa de la expresión génica de iNOS por parte de EESA 1% y 5% y de IL-6 e IL-1β por EESA 1%

Grasa abdominal:
- Ligeras mejoras en la distribución de tamaño de los adipocitos por parte de EESA 1% y 5%.
- Disminución significativa de la expresión génica de TNF- α, IL-6, IL-1β e iNOS por parte de EESA 1% y 5%

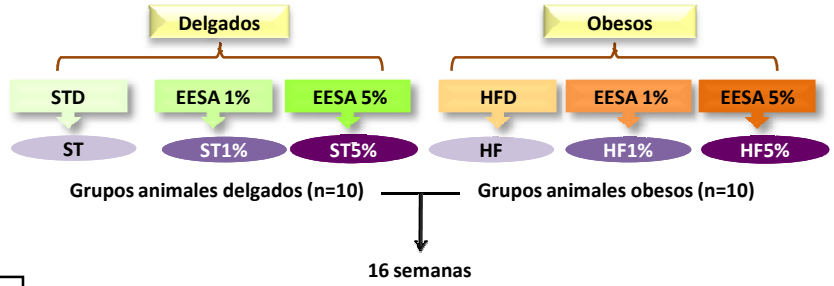
* Referidos a los animales obesos, ya que no se observaron cambios en los animales delgados debido al tratamiento.

Resumen global de resultados obtenidos en ratones con obesidad inducida por la dieta

Objetivos

Estudiar los efectos beneficiosos de una dieta alta en grasa suplementada con un extracto enzimático de salvado de arroz (EESA) en la restauración de las alteraciones asociadas a la obesidad que desarrollaron ratones C57BL/6J, a nivel cardiometabólico, adipogénico e inflamatorio.

Diseño experimental



Resultados *

Efecto cardiometabólico

- Disminución de colesterol total y glucosa en suero por parte de EESA 1%
- Disminución de los niveles de TG, insulina y adiponectina en suero por parte de EESA 1% y 5%
- Atenuación de forma concentración-dependiente de los niveles de $NO_{(x)}$ en suero por parte de EESA 1% y 5%
- Sin observarse beneficios en la tolerancia a la glucosa, se atenuó de forma significativa la resistencia a la insulina en los ratones obesos alimentados con EESA 1%

Efecto en tejido adiposo epididimal

- Mejoras en la distribución de tamaño de los adipocitos en los animales tratados, siendo esta distribución más similar al perfil de los ratones delgados en el caso de EESA 1%
- Disminución de la infiltración de macrófagos en la grasa epididimal por parte de EESA 1% y 5%, siendo este efecto mayor con EESA 1%
- Sin observarse cambios en los niveles de expresión génica de leptina en los ratones obesos, sí se dio una disminución de la expresión por parte de EESA 1% en adiponectina, TNF-α, PPARγ y Emr1, y por parte de EESA 1% y 5% en los niveles de IL-6 e IL-1β
- Respecto la expresión génica de marcadores M1/M2, EESA 1% fue capaz de disminuir significativamente la polarización de macrófagos M1, favoreciendo el equilibrio hacia los M2

Efecto en hígado

- Atenuación de los niveles de mRNA de TNF-α y Emr1 en el hígado de los animales obesos tratados con EESA 1% y 5% en comparación con los obesos controles.
- En cambio, no se observaron diferencias entre los diferentes grupos de tratamiento en los niveles de expresión génica de PPARα

* Referidos a los animales obesos, ya que no se observaron cambios en los animales delgados debido al tratamiento.

DISCUSIÓN

A lo largo de este estudio se han aportado evidencias acerca de las propiedades beneficiosas que un extracto enzimático de salvado de arroz (EESA) es capaz de ejercer sobre diferentes síntomas que se encuentran íntimamente relacionados con el síndrome metabólico. Para ello, nos hemos enfocado en el análisis de factores como la dislipemia, las alteraciones del metabolismo de la glucosa y la insulina, la disfunción vascular, el estrés oxidativo, los cambios estructurales del tejido adiposo blanco y la respuesta inflamatoria que conlleva el estado de obesidad. Se utilizaron dos modelos animales, uno con obesidad genética (ratas Zucker obesas) y otro con obesidad inducida por una dieta alta en grasa (ratones C57BL/6J que desarrollan obesidad por la dieta), con el fin de establecer el potencial terapéutico de este extracto para ser utilizado como complemento estratégico junto con la terapia normalmente aplicada al síndrome metabólico y sus complicaciones.

Conocemos como Síndrome Metabólico a un conjunto de factores de riesgo cardiovascular, entre los que se incluyen la obesidad, una glucemia elevada, dislipemias y alta presión arterial, desembocando en la mayoría de los casos en un gran riesgo de padecer diabetes tipo 2 y enfermedades cardiovasculares [29]. Los cambios en los hábitos de vida como la dieta, la pérdida de peso y hacer ejercicio de forma periódica suelen ser la estrategia de primera línea en el tratamiento del Síndrome Metabólico [2]; en cambio, existen discrepancias con respecto a la composición de la dieta óptima y la inclusión en la misma de fitonutrientes [58, 88]. Hace casi dos décadas, se descubrió que la obesidad se encuentra íntimamente asociada al desarrollo de síndrome metabólico, y a su vez, están relacionados con un estado pro-inflamatorio y pro-aterogénico que conlleva a un elevado riesgo de enfermedad cardiovascular [83]. Esta respuesta inflamatoria va a depender de la expresión de diferentes citoquinas, y además, de la movilización y uso final que tengan estos mediadores. El papel de las interleuquinas (ILs) en el metabolismo y en el mantenimiento de la homeostasis energética del organismo ha resultado ser más complejo de lo que se pensaba en un primer momento, ya que hay evidencias recientes que afirman que, para varias ILs, aunque producidas en

exceso pueden tener efectos negativos, su bloqueo o ineficacia también puede crear desequilibrios patológicos [89]. En base a esta problemática, se han desarrollado nuevas estrategias terapéuticas donde los fármacos actúan sobre la inflamación para romper el nexo de unión entre obesidad y enfermedad. A modo de ejemplo, el salsalato, un producto del salicilato, puede atenuar la actividad IKKβ/NF-κB y ha demostrado mejorar el control glucémico en pacientes con diabetes tipo 2 [90, 91]. Varios estudios pre-clínicos han estudiado los beneficios metabólicos relacionados con el estrés del retículo endoplásmico y PPARγ como dianas farmacológicas [92, 93]. Por otro lado, el bloqueo de la síntesis de TNF-α con etanercept, por ejemplo, no ha llevado a mejoras significativas en la sensibilidad a la insulina, pero sí efectos beneficiosos sobre los niveles basales de glucosa y el nivel de citoquinas pro-inflamatorias circulantes, poniéndose de manifiesto su aplicabilidad en otras enfermedades relacionadas con la obesidad [94, 95].

En paralelo, con la búsqueda de nuevas **estrategias nutricionales**, se han descubierto numerosos alimentos que presentan una composición rica en compuestos con propiedades bioactivas, y por tanto, que pueden ser aplicables en el cuidado de la salud [96]. En los últimos años se ha visto incrementado el interés por estudiar y regularizar la definición de los granos integrales de los cereales que se recomiendan con una base dietética y que son utilizados para la investigación en el ámbito de la nutrición. El primer reto aparece al definir lo que se considera grano integral, y luego definir qué es un alimento hecho a base de granos integrales. Para ello, debemos focalizarnos en que el grano integral actualmente está siendo consumido tras ser procesado, lo que constituye la base de los cuestionamientos dietéticos que aparecen en los estudios epidemiológicos y que llevan a las correspondientes recomendaciones para su consumo debido a los beneficios que supone para la salud su consumo íntegro.

Como se indicaba en la introducción de este documento, en el campo de la extracción y procesamiento del grano de arroz se han dado multitud de avances en los últimos años. Esto ha permitido, en nuestro caso, obtener un extracto que presenta una serie de ventajas frente al producto de partida, como su hidrosolubilidad, su contenido en

proteínas nutracéuticas, su diverso contenido en tocoles, y su riqueza 3,4 veces superior en γ-oryzanol [39]. En primer lugar, la actividad hipocolesterolémica de **EESA** ha sido demostrada en estudios *in vivo,* y ha sido atribuida principalmente a su contenido en γ-oryzanol*,* además de por su elevado contenido en fitosteroles. De este último grupo molecular, el esterol mayoritario en EESA es el β-sitosterol, un 4-desmetilesterol que resulta ser muy efectivo en competir con el colesterol para ser incorporado en las micelas mixtas [97]. Por otra parte, la capacidad hipocolesterolémica y antiaterogénica observada por la administración crónica de EESA puede deberse a las proteínas que lo componen, ya que la solubilidad y biodisponibilidad de las mismas en este extracto se encuentra aumentada [98]. La importancia de estas proteínas no sólo radica en su mejor asimilación por el organismo, sino en la riqueza que presentan en Arginina, aminoácido involucrado en la atenuación del proceso aterogénico y en la protección de la integridad vascular [33, 39]. Los tocotrienoles presentes en el salvado de arroz como materia original, además de destacadas propiedades antioxidantes, también actúan en la disminución del colesterol en suero, ya que son capaces de inhibir la HMG-CoA reductasa, disminuyendo por tanto la síntesis hepática de colesterol [99]. Hay que decir que moléculas relacionadas con los tocotrienoles, como el α-tocoferol, también pueden actuar como inductores de la acción de esta enzima. Se sabe que los tocotrienoles *in vivo* son transformados en parte en tocoferoles, y es por esto que se ha establecido que, aunque los tocoles en general aportan beneficios frente al estrés oxidativo y a la hiperoclesterolemia, no se debe sobrepasar cierta dosis de los mismos, ya que puede resultar contraproducente [100]. Este aspecto será comentado más adelante en profundidad.

Si queremos analizar la influencia de una dieta suplementada con EESA en el **perfil lipídico** de los animales obesos, podemos observar cómo en ratas Zucker obesas, el aumento de tejido adiposo y la resistencia a la insulina se encuentran asociados a un incremento de la lipogénesis en el hígado y a elevados niveles de ácidos grasos libres en suero, lo que conduce a la deposición de TG en el hígado junto con el aumento de los

niveles de TG y colesterol en suero [101]. En el caso de EESA, se observó una mejora de todo el perfil lipídico en los animales tratados y de forma concentración dependiente. En cambio, mientras que tuvo lugar un descenso en los niveles de TG en suero e hígado en las ratas Zucker obesas tratadas, los niveles de colesterol total fueron disminuidos en suero, pero se observó un incremento en hígado en el caso de EESA al 5%. Una explicación posible a este fenómeno podría ser la indicada anteriormente acerca de los efectos que puede tener el extracto por su contenido en tocoles a nivel hepático en un modelo donde está promovida la lipogénesis de forma genética, ya que, aunque la síntesis de colesterol se vea alterada y exista menos colesterol que alcance el riego sanguíneo, puede que se generen ciertos depósitos en el hígado cuando se trata del suplemento al 5% de EESA.

Otro aspecto característico que reproduce este modelo animal es la **hipertensión** asociada a la obesidad, que está relacionada con un descenso de la concentración de adiponectina y, a su vez, con el desarrollo de insulino-resistencia [102]. Tras el tratamiento crónico con EESA, se observaron mejoras en estos tres síntomas, siendo la dosis de EESA 5% la más efectiva. Continuando con el estudio de la función vascular, vimos que los animales obesos desarrollaron un grado moderado de disfunción endotelial, el cual fue restaurado por la dieta suplementada con EESA, especialmente por el 1% de EESA. El hecho de que las aortas de las ratas Zucker obesas tratadas con el extracto mostraran una regulación a la alza de la expresión proteica de la enzima eNOS en comparación con las ratas obesas controles, y no de su expresión génica, sugiere la posibilidad de que EESA podría inducir a una activación post-transcripcional de eNOS y, por tanto, a un aumento en la producción de NO, el principal mediador en la vasodilatación de las arterias de conductancia. Sin embargo, hay que tener en cuenta que la expresión de eNOS no proporciona información acerca de su actividad, y por ello, se utilizaron inhibidores selectivos y no selectivos de la enzima, los cuales revelaron la participación mayoritaria de esta enzima en la vasodilatación observada en las aortas de los animales obesos tratados con EESA al 1% y al 5%, y de forma concentración-

dependiente. Los resultados también demostraron que el tratamiento con EESA atenuó la hiperreactividad vascular observada en las ratas Zucker obesas frente a fenilefrina, alcanzándose en este caso un perfil de contracción similar al que presentaban los animales delgados en el caso de EESA 1%. La sobre-expresión de eNOS puede estar relacionada con esta mejora de la función vascular en el caso de la vasoconstricción de la aorta con endotelio intacto de los animales tratados con respecto a los obesos controles. Por otro lado, esta respuesta presenta un componente dependiente de endotelio importante, por el cual, se determinó la participación de las dos isoformas de NOS, mediante la inhibición selectiva y no selectiva de la enzima NOS. Se pudo determinar que la iNOS (isoforma inducible) también jugaba un papel importante en esa hiperreactividad vascular desarrollada en la aorta de las ratas obesas controles. De hecho, los resultados mostraron cómo el tejido de los animales obesos tratados presentaba una menor implicación de iNOS en la función vascular, la cual se asemejaba a la de las ratas delgadas. La mejora que introduce EESA tanto a nivel vascular como del endotelio podría ser la clave de la mejora que genera la administración del extracto cuando coexiste una hipertensión moderada de forma previa en estos animales obesos.

La producción excesiva de NO procedente de la enzima iNOS ha demostrado tener consecuencias metabólicas como la reducción del consumo de glucosa mediado por insulina y la inducción de estrés celular y muerte de las células β-pancreáticas en animales obesos. En el caso de nuestro tratamiento, se observó que el aumento de la expresión vascular de iNOS que presentaba el grupo obeso control frente al delgado fue restaurado en los animales tratados. Por tanto, se puede deducir que las complicaciones que pueden derivarse del aumento de la implicación del NO dependiente de iNOS pueden ser prevenidas mediante una dieta suplementada con EESA. De forma paralela, la sobre-expresión de iNOS está íntimamente relacionada con una sobre-expresión de TNF-α, tal como se pudo observar en la expresión vascular de ratas Zucker obesas. Este hecho, unido a la disminución en los animales obesos tratados de los niveles de nitritos

en suero, denota una disminución del grado de inflamación previo a otras patologías que se observa en este modelo animal al final de tratamiento.

Otro aspecto ineludible en la obesidad y en el síndrome metabólico como factores de riesgo cardiovascular es el **estado oxidativo** de los órganos y tejidos de los animales obesos. Se ha demostrado que tanto el γ-oryzanol como los demás compuestos con potencial antioxidante que se encuentran en EESA tienen una importante capacidad protectora frente a la presencia elevada de ROS, inhibiendo a su vez la acción de citoquinas pro-inflamatorias y aumentando los niveles de adiponectina en suero en ensayos *in vitro* e *in vivo*. La actividad antoxidante del γ-oryzanol, como se ha indicado anteriormente, se le atribuye tanto a la molécula que lo conforma, el ácido ferúlico, como a los esteroles con los que se encuentra esterificado en las células vivas [103]. En nuestro estudio, el elevado contenido de EESA en estas sustancias juega un papel fundamental en el desarrollo de su actividad antioxidante sobre la pared vascular de las ratas obesas. Este potencial se confirmó al observar que en los animales tratados con el extracto se veía incrementada la expresión génica de las subunidades NOX-1 y $p22^{phox}$ de la NADPH oxidasa, mientras que en los animales obesos sin tratar existió un aumento significativo con respecto al grupo delgado control, lo que puede ser en parte origen de la disfunción vascular y la hipertensión moderada que presentaban estos animales.

Por otro lado, cuando se investigaron los efectos de la dieta con EESA en los **microvasos** se observó que, aunque no se vio afectada la vasorelajación a acetilcolina dependiente de endotelio por parte de los grupos experimentales, tuvo lugar una disminución de la contribución de NO en el mecanismo de vasodilatación que siguieron las arterias de resistencia de las ratas Zucker obesas controles. En cambio, los animales tratados con EESA al 5% vieron incrementada su capacidad vasodilatadora hasta niveles similares a los de los animales delgados controles cuando se encontraban en presencia de inhibidores de las vías moleculares independientes de óxido nítrico como pueden ser inhibidores de la ciclooxigenasa o del EDHF, lo que nos lleva a suponer que, efectivamente, existe en estos animales una activación de la eNOS. Varios estudios

experimentales sugieren que la contribución de los EDHF aumenta cuanto más disminuye el calibre del vaso arterial, experimentándose una actividad predominante en los vasos de resistencia y una regulación incrementada como mecanismo de compensación frente a situaciones de reducida biodisponibilidad de NO [104]. Por esta razón, la recuperación además del papel desarrollado por los EDHF en las arterias de los animales obesos tratados con EESA puede ser una vía alternativa por las que se contrarresta la pérdida del óxido nítrico disponible que tiene lugar en estos animales. A pesar del debate que rodea a la identidad del EDHF, se acepta de forma general como dilatación dependiente de los mismos aquella que depende de los canales de potasio dependientes de calcio de baja (SK_{Ca}) e intermedia (IK_{Ca}) conductancia. En las ratas Zucker obesas está descrito un deterioro de los EDHF, ya que se ven afectados en los desórdenes metabólicos, especialmente en las alteraciones de la glucosa y de la insulina [105]. En nuestro estudio, la dieta enriquecida con EESA indujo un incremento de la expresión de los canales SK_{Ca} en los animales tratados frente a los obesos no tratados, alcanzando niveles de expresión cercanos a los del grupo delgado. Además, el grupo alimentado con la dieta suplementada al 5% de EESA mostró un aumento significativo en la expresión de ambos canales, SK_{Ca} e IK_{Ca}. Estos resultados concuerdan con la mejora de la insulino-resistencia que observamos en las ratas Zucker obesas tratadas en comparación con sus controles. Como se ha indicado anteriormente, en el animal obeso tiene lugar un bloqueo o deterioro de los canales SK_{Ca} e IK_{Ca}, lo que tiene consecuencias a nivel del vaso arterial, ya que la hiperpolarización necesaria para la vasorrelajación se ve interrumpida, traduciéndose en una despolarización de la membrana celular, y a nivel del endotelio vascular, ya que debido a la despolarización, aumenta la producción de ROS y disminuye la biodisponibilidad del óxido nítrico. En el caso de nuestro tratamiento, se observó cómo la dieta complementada con EESA produjo una atenuación significativa del estrés oxidativo vascular, lo que nos lleva a pensar que existe una relación entre esta disminución de ROS y la recuperación del papel de los EDHF.

Hay evidencias claras de que la patogénesis de la resistencia a la insulina asociada a la obesidad y la diabetes tipo 2 incluye un grado moderado de **inflamación** y una activación del sistema inmune. Los marcadores inflamatorios a nivel sistémico contribuyen entonces como factores de riesgo al desarrollo del estado de diabetes y a las complicaciones vasculares que la acompañan. El tejido adiposo, el hígado, el músculo y el páncreas se transforman por sí mismos en lugares desde donde radica esta inflamación en el estado de obesidad. En estos tejidos se da una infiltración de macrófagos y otras células inmunes donde conviven células con un perfil pro- o anti-inflamatorio [21]. Estas células van a ser cruciales para la producción de citoquinas inflamatorias, ya que actúan de forma autocrina y paracrina y van a interferir en los procesos de señalización de la insulina en los tejidos periféricos o provocando alteraciones en las células β con la subsecuente deficiencia insulínica. Varias son las citoquinas involucradas en el desarrollo del proceso inflamatorio, como por ejemplo, la IL-6, la cual se ha detectado una relación dosis-respuesta significativa entre su aumento y el riesgo de diabetes tipo 2 [106]. Tanto la obesidad como la insulino-resistencia, dos aspectos cardinales del síndrome metabólico, se encuentran unidos, como se ha dicho al comienzo del texto, a un grado moderado de inflamación [107]. Se sabe que el tejido adiposo blanco es el lugar donde radica este proceso patológico, y como consecuencia, da lugar a una serie de cambios morfológicos y funcionales en el mismo. Entre otras cosas, los adipocitos se ven modificados en tamaño, y también se ve afectada su distribución en el tejido según el tamaño. En el sujeto obeso, los adipocitos liberan mayor cantidad de ácidos grasos libres y quimioquinas, seguido de una infiltración de macrófagos que, como hemos dicho, van a provocar un incremento de la producción de IL-1β, IL-6 e iNOS [108, 109].

Como se ha indicado previamente, **los componentes funcionales de la fracción lipídica** de EESA incluyen fitosteroles, γ-oryzanol, tocoferoles y tocotrienoles. Estos últimos, al igual que los polifenoles, han demostrado tener **propiedades antioxidantes y anti-inflamatorias**, a la vez que actividad frente a enfermedades crónicas como el cáncer, la

diabetes, las enfermedades cardiovasculares y algunos desórdenes neurológicos [110]. Además, se ha visto que el γ-tocotrienol es capaz de mejorar las alteraciones funcionales relacionadas con la obesidad que se generan en los adipocitos atenuando la expresión de adipoquinas pro-inflamatorias en células 3T3-L1 [111]. Por otro lado, el componente bioactivo principal del extracto objeto de nuestro estudio es el γ-oryzanol, al que se han atribuido efecto anti-hiperlipidémico, anti-inflamatorio, anti-cáncer y antioxidante. Estudios *in vivo* e *in vitro* han demostrado cómo el γ-oryzanol es capaz de ejercer su efecto anti-inflamatorio mediante la regulación a la baja de un factor de transcripción, el factor nuclear κβ (NF-κβ) que, a su vez, disminuye la expresión de enzimas inflamatorias como ciclooxigenasa 2 o iNOS, y de citoquinas pro-inflamatorias como IL-1β, IL-6 y TNF-α [112]. En nuestro caso, los estudios realizados han demostrado que una dieta enriquecida con EESA y administrada a un modelo genético de síndrome metabólico como son las ratas Zucker obesas, es capaz de disminuir la expresión génica de citoquinas como TNF-α, IL-6, IL-1β e iNOS, aunque de diferente manera según el tejido adiposo del que se trate, ya que las mejoras que el tratamiento con EESA es capaz de producir a estos niveles parece que es independiente de la dosis utilizada en el caso de la grasa abdominal, y selectivas para el 1% de EESA en el caso de la grasa epididimal, considerada la más representativa para la mayoría de los autores para medir la expresión de mediadores inflamatorios [113, 114]. Según los resultados obtenidos con EESA en ratones DIO como otro modelo de obesidad, efectivamente una dieta complementada con EESA al 1% demuestra un elevado potencial para restaurar de forma significativa los niveles de mRNA de marcadores como adiponectina, PPARγ, TNF-α y Emr1 en el tejido adiposo epididimal. A su vez, ambas dosis del extracto ejercieron el mismo efecto en la expresión génica de IL-1β e IL-6, disminuyéndola significativamente en comparación con los niveles de expresión observados en los controles obesos.

Aunque el TNF-α ha sido considerado en los últimos años la clave en el campo de la inmunidad y en los procesos inflamatorios, también fue imprescindible la identificación de NF-κB, una familia de factores de transcripción cuya activación es responsable de la

inducción de genes pro-inflamatorios e implicados en la inmunidad innata [115]. En base a los resultados obtenidos a lo largo de este estudio en relación a los efectos de EESA sobre los mediadores de la inflamación asociada a la obesidad en diferentes modelos animales, podríamos decir que **el extracto ejerce su actividad** mediante dos vías: una inflamatoria, en la que posiblemente el componente más implicado sea el γ-oryzanol, el cual actúa inhibiendo el NF-κB y, por tanto, disminuyendo la expresión de TNF-α a diferentes niveles; y otra vía, que podríamos considerar oxidativa, dependiente de los niveles de ROS, y que se puede manifestar en los niveles de nitritos en sangre o en la expresión de iNOS en los diferentes casos. Esto explicaría por qué, cuando la activación de una de estas dos vías no se ve atenuada por el tratamiento, como es el caso de la grasa epididimal en las ratas Zucker, donde la expresión de TNF-α no sufre modificaciones por el extracto, sólo en la que parece ser su concentración óptima (EESA 1%) tiene efectos sobre IL-1β e IL-6. En cambio, en los casos donde tanto la vía inflamatoria (TNF-α) como la oxidativa (ROS o derivados del NO) parecen estar afectadas de forma positiva por el extracto, como parece ocurrir en el caso de los ratones DIO alimentados con el suplemento de EESA, IL-1β e IL-6 aparecen disminuidas indistintamente de la concentración utilizada. En este aspecto, serían necesarias algunas pruebas para valorar cómo ha influido el tratamiento con EESA en el estrés oxidativo vascular que se haya podido generar en las arterias de los ratones DIO, y poder hacer una comparativa de los resultados observados en el modelo genético de síndrome metabólico.

Por último, otra cuestión que surge tras la visión global de los resultados del estudio es la razón de por qué el extracto parece ejercer su acción, no de un modo concentración-dependiente, sino de forma más bien selectiva. El uso de EESA 5% parece ser más efectivo en aspectos como la vasodilatación mediada por NO o por el EDHF, y en el caso de las ratas Zucker puede estar relacionado con la restauración que produce a nivel de adiponectina, insulino-resistencia y nitritos. En cambio, se observa según la bioquímica en suero y los parámetros generales del modelo de obesidad inducida por la dieta, que

EESA 1% parece prevenir las alteraciones del perfil lipídico y frenar notablemente la remodelación del tejido adiposo en los ratones obesos tratados. Por otro lado, y como queda indicado en el primero de los estudios, podemos intuir que EESA 5% puede conllevar con su administración a cierto desbalance ventajas-riesgos en lo que al metabolismo lipídico se refiere, ya que su elevado contenido en tocoles, los cuales le aportan en buena medida su propiedad antioxidante en sinergia con otros de sus compuestos bioactivos, pueden producir a partir de una cierta cantidad un viraje metabólico de tocotrienoles a tocoferoles que actúen de forma contraproducente sobre la HMG-CoA, pasando de inhibir la síntesis de colesterol (a la vez que su absorción por el efecto de los fitosteroles y el γ-oryzanol) a inducirla [116]. Otra posible explicación a esta respuesta independiente de la dosis que presenta el extracto podría ser paralela a los efectos adversos que presentan algunos fármacos utilizados normalmente para la diabetes tipo 2 como las glitazonas, las cuales, tienen como mecanismo de acción uno de los observados con EESA: la activación de PPARγ. Se ha observado que las glitazonas sobre los lípidos reducen los niveles de triglicéridos y ácidos grasos libres, y aumentan el colesterol total, el HDL-c y el LDL-c (aunque parece que aumenta el tamaño de las partículas y las hace menos densas, y por tanto, menos aterogénicas) [117]. Estas pueden ser razones que expliquen esa "frontera" de seguridad en lo que respecta a la **concentración óptima** para que EESA ejerza sus efectos beneficiosos. Aun así, dependerá también del modelo animal utilizado, ya que en el caso de los ratones DIO, EESA muestra beneficios a todos los niveles, al 1% y al 5%, pero de una manera más específica, destacando su actividad sobre la estructura del tejido adiposo, la distribución de tamaño de los adipocitos y la expresión génica, no sólo de mediadores inflamatorios, sino también de mediadores con influencia metabólica como PPARγ, con un papel trascendente en la polarización de macrófagos M1/M2, y a nivel de hígado, como otro de los órganos especialmente afectados en el proceso inflamatorio.

A la luz de los resultados obtenidos durante todo el estudio, podemos concluir que la administración crónica de EESA al 1% y al 5% junto con el alimento es capaz de actuar

de forma preventiva en varios aspectos de la patología del síndrome metabólico, ralentizando el desarrollo e instauración de dislipemias, de desórdenes relacionados con el metabolismo de la glucosa y la insulina, de alteraciones vasculares, y de cambios estructurales y funcionales del tejido adiposo como órgano especialmente involucrado en el proceso inflamatorio, todos ellos factores agravantes del estado de obesidad.

Interés de EESA como complemento ideal en la prevención y tratamiento del Síndrome Metabólico.

En el presente trabajo se pone de manifiesto la importancia de este extracto como alimento funcional aportando datos decisivos acerca de su **eficacia y aplicabilidad** mediante su administración en diferentes modelos animales de obesidad y Síndrome Metabólico. En vista de los resultados observados, parece interesante plantear estudios futuros donde se estudien los beneficios de la suplementación de EESA mediante ensayos clínicos, enfocados en pacientes con sobrepeso, obesidad, Síndrome Metabólico o con un elevado riesgo cardiovascular. De esta manera, podríamos llegar a la conclusión de si realmente este complemento nutricional puede aportar un elemento novedoso dentro de la amplia gama de nutracéuticos que hoy día coexisten en el mercado (Fig. 7). En nuestro caso, y en base a los estudios previos, se apuesta por un producto versátil en cuanto a aplicabilidad terapéutica frente a diversas patologías, rentable a nivel económico y cuyo consumo sería fácil de introducir en la vida cotidiana (Fig. 7).

En la actualidad, la oferta de medicamentos que existe para los pacientes que sufren síndrome metabólico o patologías relacionadas para prevenir o reducir la ingesta calórica se reduce a inhibidores de las lipasas (Orlistat), anorexígenos con acción sobre el sistema nervioso central (combinación fentermina/topiramato, lorcaserina) o antidepresivos (Bupropión, fluoxetina). Es decir, se trata de un grupo de pacientes que normalmente ya siguen un tratamiento y que, además, se ven obligados a medicarse

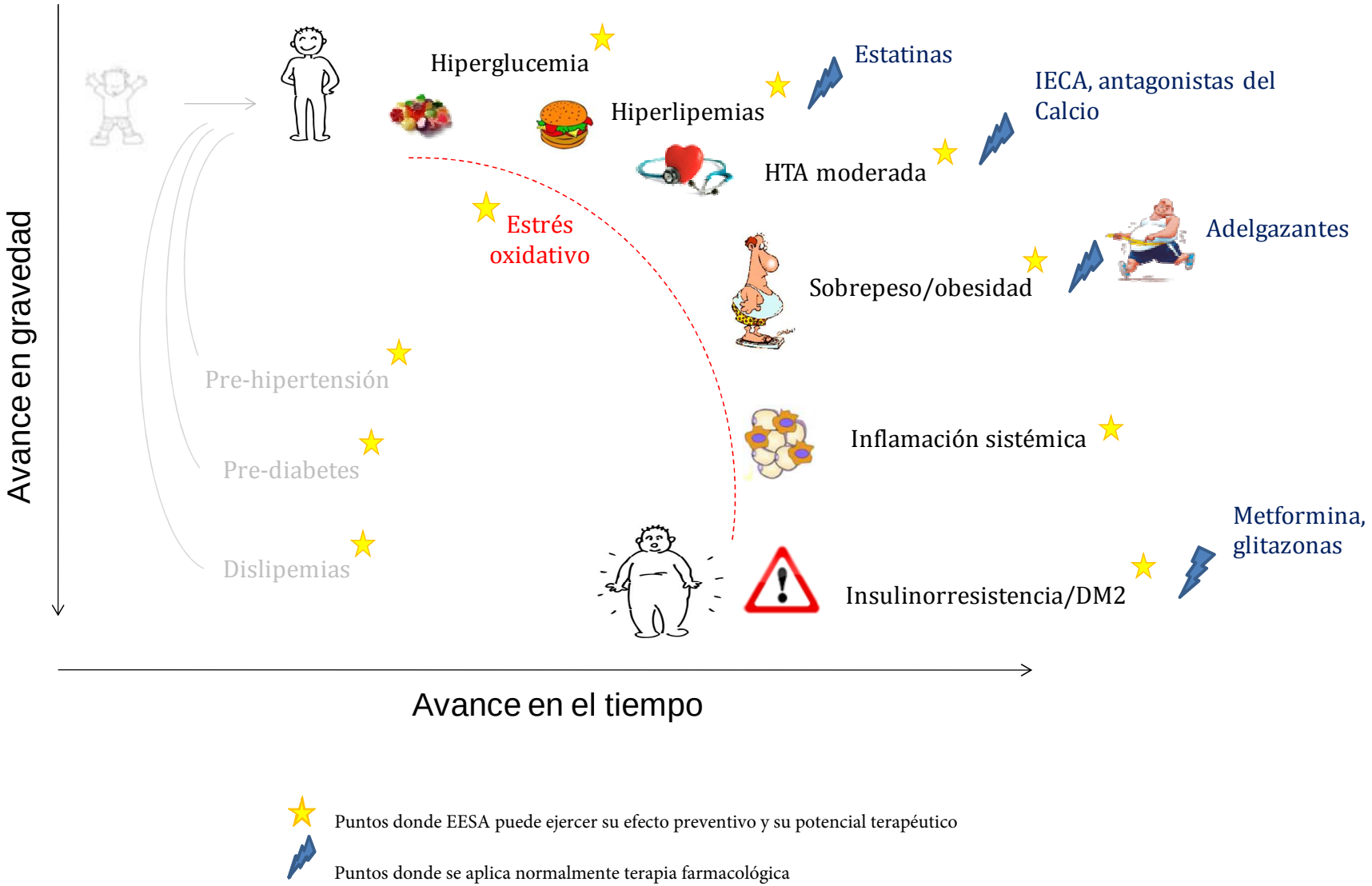
también para controlar el apetito y/o facilitar la eliminación de grasas de una forma no fisiológica. En cambio, está ampliamente demostrado que a pesar de estas terapias farmacológicas, los malos hábitos del paciente y la falta de conocimiento en lo que a alimentación se refiere son los mayores factores de riesgo de enfermedad cardiovascular en los adultos. En el caso de los niños, se podría decir que la **prevención** adquiere una importancia crítica, ya que se ha observado que en el 50 % de los niños entre los 10 y los 14 años presentan ciertas alteraciones asociadas a la obesidad, a la diabetes o a la aterosclerosis cuya progresión y consecuencias dependerán exclusivamente de los hábitos nutricionales que estos niños hayan adquirido [118].

Por tanto, la única **terapia coadyuvante** que puede ser efectiva y viable en el tiempo es aquella que obligue y ayude al paciente a mejorar sus hábitos alimenticios, tomar conciencia de su salud y no depender de fármacos que puedan complicar o reducir su calidad de vida. Desde este punto de vista, podría ser interesante apostar por EESA como un candidato ideal en la prevención desde la infancia o como coadyuvante del tratamiento de diferentes patologías de nuestros días como puede ser la obesidad, la diabetes, la hipertensión o las dislipemias [119] (Fig. 7). De hecho, en la última década se ha incrementado de forma notoria el interés por parte de la población de las terapias complementarias, especialmente en el ámbito de medicina natural y los suplementos dietéticos. Este giro en los hábitos de consumo con respecto a los productos de uso terapéutico puede deberse a varios factores, entre ellos las creencias personales de los pacientes, el incremento de publicidad en los medios de comunicación o los propios cambios de actitud de la sociedad.

En el caso de las patologías relacionadas con la obesidad, hay autores que han profundizado en el estudio del uso de la medicina complementaria y alternativa, incluyendo el uso de suplementos nutricionales, por parte de **los pacientes** que tienen un riesgo elevado de padecer síndrome metabólico y los que no presentan riesgo alguno. La conclusión es que el grupo de población susceptible de padecer síndrome metabólico tiende cada vez con mayor frecuencia a utilizar la medicina complementaria

y alternativa, y a menudo, suplementos nutricionales. La explicación puede ser que estos pacientes, al sufrir problemas de salud complejos que exigen normalmente estar multimedicados, suelen tener una sensación de poco éxito en el tratamiento de su salud mediante el uso de los medicamentos convencionales. De esta forma, también se ha demostrado que los pacientes que sufren enfermedades crónicas también suelen acudir a las terapias alternativas, o los pacientes incluso con diabetes tipo 1 se observa que confían cada vez más en el uso de plantas medicinales como coadyuvantes para el control de la glucemia. El problema que radica en los hábitos de estos pacientes con patologías asociadas a la obesidad es que éstas tienen un carácter multifactorial y diverso en sus causas, y por tanto, quizás el remedio también debería poderse tratar desde diferentes vertientes para optimizar el tratamiento y aportar calidad de vida al individuo. Sin embargo, esta labor se ve limitada debido a que precisamente el grupo poblacional que suele incluir este tipo de terapias y suplementos nutricionales a sus hábitos no suele comunicárselo a su médico especialista o a los profesionales sanitarios que le atienden normalmente. Estos hechos deberían hacernos reflexionar a todos los profesionales sanitarios acerca de por qué los pacientes temen hacer partícipe al profesional sanitario de sus preferencias terapéuticas, ya que sabemos que existen numerosos efectos adversos que pueden darse como consecuencia de un uso inadecuado de la **combinación de la terapia convencional con la natural**. Esta complicación es fácilmente salvable siempre que los responsables de la salud seamos conscientes de la importancia o trascendencia que puede tener tanto ejercer una buena labor preventiva (como puede suponer para un niño con riesgo elevado de padecer patologías relacionadas con el síndrome metabólico), como conocer todas las opciones de terapia que podemos ofrecer a los pacientes.

Fig. 7 Posible proceso patológico que puede desembocar en Síndrome Metabólico y puntos de acción de EESA.



CONCLUSIONES

El conjunto de resultados obtenidos sugiere que una dieta suplementada con EESA ejerce efectos beneficiosos frente al desarrollo del Síndrome Metabólico y sus complicaciones cardiovasculares y metabólicas asociadas.

Por tanto, en base a los objetivos que nos propusimos al comienzo del estudio, podríamos concluir lo siguiente:

- Una dieta suplementada con 1% y 5% de EESA es capaz de atenuar la hipertensión moderada, prevenir la resistencia a la insulina y mejorar el perfil bioquímico asociado al síndrome metabólico desarrollado en ratas Zucker obesas de forma dosis-dependiente.

- Paralelamente, el tratamiento con EESA 1% y 5% fue capaz de producir mejoras frente a las alteraciones vasculares observadas en la aorta de los animales obesos, ya que atenuó la disfunción endotelial y la hiperreactividad vascular mediante un aumento de la expresión proteica de eNOS, disminuyó el estrés oxidativo vascular actuando sobre la producción de anión superóxido y la expresión de subunidades de la NADPH oxidasa, y redujo el grado de inflamación vascular generada por la expresión de iNOS y TNF-α

- Respecto a los efectos de una dieta enriquecida con EESA sobre la microvasculatura, se observó que el tratamiento al 1% y al 5% era capaz de reducir las alteraciones observadas en las arterias del lecho mesentérico de ratas Zucker obesas mediante la disminución de la producción de radicales libres de oxígeno, el aumento de la producción y liberación de NO y la restauración de la implicación del EDHF en la vasodilatación.

- La administración del extracto logró atenuar los cambios estructurales del tejido adiposo que acompañan a la obesidad tanto en ratas Zucker obesas como en ratones DIO. En el caso del modelo genético de ratas Zucker, ambas concentraciones de EESA actuaron significativamente frente a la sobre-expresión de mediadores inflamatorios como TNF-α, IL-6, IL-1β e iNOS en la grasa abdominal, y de forma parcial en la grasa epididimal. En los ratones DIO, 1% EESA actuó como modulador de la respuesta inflamatoria asociada al síndrome metabólico, influyendo en la producción de citoquinas y en la polarización de macrófagos.

- Por tanto, de forma general podemos considerar que EESA podría ser una alternativa ideal en el campo de los alimentos funcionales, ya que podría ser utilizado como coadyuvante en la profilaxis de las complicaciones asociadas a la obesidad o como complemento dietético junto con las terapias aplicadas al síndrome metabólico.

ABREVIATURAS

A

1400W: inhibidor de la enzima óxido nítrico sintasa inducible

AA: ácido araquidónico

AC: adenil ciclasa

ACh: acetilcolina

AF: ácido ferúlico

AG: ácidos grasos

α: receptor alfa-adrenérgico

C

COX: cicloooxigenasa

CV: cardiovascular

CYP7A1: citocromo P450 7A1

$[Ca^{2+}]i$: concentración de calcio intracelular

D

DAG: diacil-glicerol

DSS: *dextrano sulfato sódico*

E

EDHF: factor hiperpolarizante dependiente de endotelio (*endothelium dependent hyperpolarizing factor*)

EESA: extracto enzimático de salvado de arroz (=RBEE)

Emr1: gen codificador de una proteína transmembrana presente en la superficie de los macrófagos

eNOS: óxido nítrico sintasa endotelial (*endotelial nitric oxide synthase*)

F

FceR1: gen del receptor de inmunoglobulina E de alta afinidad

Fen: fenilefrina

G

GC: guanilil ciclasa

GMPc: guanosín monofosfato cíclico

H

HDL-c: colesterol transportado en lipoproteínas de alta densidad (*high density lipoprotein cholesterol*)

HF: animals alimentados con dieta alta en grasa

HF1%: animales alimentados con comida alta en grasa suplementada con EESA 1 %

HF5%: animales alimentados con comida alta en grasa suplementada con EESA 5 %

HFD: comida alta en grasa (*high fat diet*)

HMG-CoA: 3-hidroxi-3-metilglutaril-coenzima A

HOMA-IR: índice homeostático de evaluación de la resistencia en insulina

I

ICAM: proteína de adhesión intercelular (*intercelular adhesion molecule)*

IFN-γ: interferón-gamma

IgE: inmunoglobulina E

IK_{Ca}: canales de potasio dependientes de calcio de conductancia intermedia (*intermediate conductance calcium activated potassium channels*)

IKKβ: IκB quinasa β

IL-1β: interleuquina 1β

IL-6: interleuquina 6

ILs: interleuquinas

INDO: inhibidor de la enzima ciclooxigenasa (indometacina)

iNOS: óxido nítrico sintasa inducible (*inducible nitric oxide synthase*)

L

L= LC =LZ: animales delgados controles

L1: animales delgados alimentados con EESA 1 %

L5: animales delgados alimentados con EESA 5 %

LDL-c: colesterol transportado en lipoproteínas de baja densidad (*low density lipoprotein cholesterol*)

L-NAME: inhibidor de la enzima óxido nítrico sintasa (*L-NG-nitroarginine methyl ester*)

LPS: lipopolisacárido

M

M: receptor muscarínico

M1: macrófagos activados de forma clásica (pro-inflamatorios)

M2: macrófagos activados de forma alternativa (anti-inflamatorios)

MMP-2: *matrix metalloproteinase-2*

mRNA: ARN mensajero

N

NADPH: nicotinamida adenina dinucleótido fosfato

NF-κβ: factor nuclear κβ (*nuclear factor κβ*)

NK: células *natural killers*

$NO_{(x)}$: nitritos

NO: óxido nítrico (*nitric oxide*)

NOS: óxido nítrico sintasa (*nitric oxide synthase*)

O

O= OC =OBZ: animales obesos controles

O1 = O1% = OB1: animales obesos alimentados con EESA 1 %

O5 = O5% = OB5: animales delgados alimentados con EESA 5 %

OMS: Organización Mundial de la Salud

P

PAS: presión arterial sistólica

PCR: reacción en cadena de la polimerasa

PGI_2: prostaglandina I_2

PPAR-γ: receptor gamma activado por proliferador de peroxisomas (*peroxisome proliferator-activated receptor-gamma*)

PPARα: receptor alfa activado por proliferador de peroxisomas (*peroxisome proliferator-activated receptor-alpha*)

PVAT: tejido adiposo perivascular (*perivascular adipose tissue*)

R

RBEE: *rice bran enzimatic extract* (=EESA)

RLO: radicales libres de oxígeno

ROS: especies reactivas de oxígeno (*reactive oxygen species*)

S

SK_{Ca}: canales de potasio dependientes de calcio de baja conductancia (*small conductance calcium activated potassium channels)*

ST: animales alimentados con comida estándar

STD: comida estándar (*standard diet*)

T

TG: triglicéridos

TNF-α: factor de necrosis tumoral-alfa (*tumor necrosis factor-alpha)*

TXA: tromboxano A

U

UVB: ultravioleta B

V

VCAM: proteína de adhesión a la célula vascular *(vascular cell adhesion molecule)*

VEGF: factor de crecimiento del endotelio vascular (*vascular endothelium growth factor*)

VLDL-c: colesterol transportado en lipoproteínas de muy baja densidad (*very low density lipoprotein cholesterol*)

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CHARACTERIZATION OF THE FUNCTIONAL PROPERTIES OF A RICE BRAN ENZYMATIC EXTRACT ON CARDIOVASCULAR DISEASES AND METABOLIC SYNDROME

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Characterization of the functional properties of a rice bran enzymatic extract on cardiovascular diseases and Metabolic Syndrome

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Up to 25 percent of the world's adult population is estimated to have the **metabolic syndrome**, a condition closely associated with central obesity. The metabolic syndrome is a major risk factor for cardiovascular disease and type 2 diabetes and therefore represents an important health problem worldwide. In addition to metabolic abnormalities such as raised fasting plasma glucose, high cholesterol and high blood pressure, there is consensus that obese subjects develop a state of low grade chronic immune activation. This sustained pro-inflammatory response in fat tissue is thought to worsen insulin resistance and dyslipidemia [1]. Moreover, overweight and obesity are associated with a profound **endothelial dysfunction**, which is thought to contribute etiologically to the development of other vascular alterations and the increased risk of cardiovascular disorders [2]. Although the fat mass could be considered damaging for tissues functionality, it has been demonstrated the possible contribution of perivascular adipose tissue to the regulation of vascular tone and remodeling. In the healthy lean state, adiponectin and adventitium-derived relaxing factors are released by perivascular fat to decrease contractile responses to vasoconstrictive agents, thus exerting a protective anti-hypertensive function via the control of vasodilation [3].

In obesity, **white adipose tissue** (WAT) is remodeled dynamically by adipocyte hypertrophy (increased size), hyperplasia (increased number), immune cell infiltration, endothelial cell overactivation, and extracellular matrix overproduction. This remodeling may trigger metabolic and hypoxic stress, resulting in activation of multiple inflammatory signaling pathways, ultimately leading to dysregulation of numerous adipokines including **proinflammatory cytokines,** chemokines, growth factors, acute-phase proteins, and complement-like factors. Such a deregulation is the main feature of adipose tissue low-grade inflammation and contributes to the pathogenesis of metabolic syndrome [4]. On the other side, obesity also compromises metabolic homeostasis by altering **glucose absorption and insulin sensitivity** in liver, skeletal muscles, and fat tissues. Indeed, the systemic inflammation associated to metabolic syndrome development starts in WAT. Here, adipocytes and immune cells trigger adipose tissue

inflammation, which affects: the vasculature increasing permeability of **endothelium**, thereby triggering vessels recruitment, plaque development and cardiovascular disease, and the anabolic actions of **insulin**, causing insulin resistance and consequently, hyperinsulinemia, hyperglycemia, and hyperlipidemia. Besides, the chemokine system links obesity to insulin resistance by regulating **macrophages** functional responses as well as by controlling pro-inflammatory monocyte influx in WAT [5]. The obesity-induced macrophage phenotype switch in expanding adipose tissue involves a decrease in anti-inflammatory M2 macrophages paralleled with an increase in pro-inflammatory M1 macrophages content [6].

Whole grains have been hypothesized to reduce the risk of type 2 diabetes based on their content of fiber, vitamins and minerals and phytochemicals which may improve insulin sensitivity and glucose metabolism, and also by reducing overweight and obesity [7]. **Rice bran**, the brown outer layer of rice kernel, is mainly composed of pericarp, aleurone, subaleurone layer, and germ. It contains appreciable quantities of nutrients like protein, fat, and dietary fiber. Furthermore, it contains substantial amount of minerals like K, Ca, Mg, and Fe. The presence of high-quality proteins and antioxidants such as tocopherols, tocotrienols and **γ-oryzanol** also brighten prospects of rice bran utilization for humans as a functional ingredient to mitigate the life-threatening disorders. [8]. Despite these nutraceutical advantages that rice bran provides, rice husks have been considered inedible, because of the natural insolubility of its proteins and its high content in liposoluble compounds. This is the reason why rice bran has various non-food applications, such as low-value waste materials [9]. These limitations have caused that in the last few years, rice bran oils have been widely used in the investigation of its properties [10, 11]. However, these extracts showed other disadvantages such as the risk of rancidity and a limited bioavailability of its components, which has been ameliorated through other processes of extraction [12, 13]. In this context, a new rice bran enzymatic extract (**RBEE**) was obtained, which has an appearance of brown syrup and presents several advantages facing the raw material: its water-solubility, which

confers to the product an easier maintaining, manipulation and administration; its high content in nutraceutical, soluble and bioavailable proteins, because of its low molecular weight; and its intact content in liposoluble compounds such as poly-unsaturated fatty acids, vitamin E and γ-oryzanol (a mixture of 10 ferulate esters of triterpene alcohols), which is in RBEE 3,4-fold that in the original rice bran [14].

As we have indicated above, different beneficial properties have been attributed to the interesting composition of rice bran. Some authors have explained how diets rich in polyphenols, which are more easily assimilated with food than administered alone, have preventive activity against the development of **cardiovascular diseases** [15]. At the same time, it has been evidenced that these compounds preserve nitric oxide and ciclooxygenase-derived mediators releasing in deteriorated arteries, and favor endothelium-derived hyperpolarizing factor (EDHF) response [16]. Other studies have highlighted the possibilities of rice bran components to be used in the prevention of vascular diseases, since ferulic acid (which takes part of γ-oryzanol molecules) is able to exert beneficial actions in hypertension, dyslipidemias and hyperglycemia developed in obese rats [17, 18]. Furthermore, benefits in deregulation of **glucose homeostasis** have been shown by the administration of whole rice grain, because of its content in fiber and other phytonutrients with a highly antioxidant capability [19, 20]. On the other side, the effects of rice bran intake in **dyslipidemias** have been classically studied; since its content in γ-oryzanol, tocols and phytosterols makes that rice bran could be useful in the amelioration of lipid profile in obese subjects [21-23]. Finally, within rice bran properties, **anti-inflammatory activity** has also been widely investigated. On this regard, polyphenols, arabinoxilan, and γ-oryzanol seem to be the responsible molecules of this action [24-26].

In our case, RBEE could provide vascular benefits, since it is a good source of the aminoacid Arginin, the precursor of nitric oxide, which represents the 13% of the extract protein content [27]. Moreover, several investigations show that RBEE have antioxidant, anti-hyperlipidemic and anti-inflammatory effects [14, 26] together with

anti-proliferative and skin protective activities [12, 28]. These findings have encouraged us to determine what could be the potential of RBEE to be used a functional food in the therapy of metabolic syndrome and its associated complications.

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The general aim of this project have been the study of the beneficial properties that a water-soluble rice bran enzymatic extract (RBEE) provides against metabolic and cardiovascular alterations associated to the development of Metabolic Syndrome in animal models of obesity.

In order to get this purpose, the following objectives were established:

- To determine if the administration of a 1 % and 5 % RBEE-supplemented diet was able to attenuate moderate hypertension as well as glucose metabolism and lipid profile disorders related to Metabolic Syndrome in obese Zucker rats.
- To evaluate the effects of a RBEE-enriched diet on vascular and endothelial function in aorta from obese Zucker rats. The main molecular mechanisms involved in these effects were also investigated, particularly those related to vascular oxidative stress and inflammation.
- To study if RBEE was able to restore microvascular alterations in obese Zucker rats, one of the earliest predictive cardiovascular risk factor, and the mechanisms underlying this action. Specifically, the contribution of nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and reactive oxygen species (ROS) to the microvascular activities of RBEE was studied.
- To investigate the effects of a RBEE-treatment over morphological and functional changes in the adipose tissue of obese Zucker rats. We determined the involvement of rice bran supplementation on the expression of inflammatory biomarkers in white adipose tissue of the genetic animal model of obesity.
- To investigate the therapeutic activity of long-term administration of RBEE in mice with a high fat diet-induced obesity, focusing our attention in glucose metabolism, biochemical profile and adipose tissue morphology and function associated to obesity. The effect of RBEE on macrophages phenotypes and pro-inflammatory genes expression in white adipose tissue was determined.

All together our results suggest that RBEE exerts beneficial effects in the development of metabolic syndrome and its associated cardiovascular and metabolic complications.

Therefore, after the development of these thesis objectives we might conclude:

- 1% and 5% RBEE-supplemented diet was able to attenuate moderate hypertension, prevent insulin resistance and ameliorate the biochemical profile associated to metabolic syndrome developed in obese Zucker rats in a concentration-dependent manner.

- Moreover, treatment with 1% and 5% RBEE was able to produce ameliorations facing the vascular disorders observed in the aorta of obese animals in different levels, attenuating endothelial dysfunction, vascular hyperreactivity and inducing an enhanced eNOS expression; decreasing vascular oxidative stress acting over the superoxide anion production and the expression of NADPH oxidase subunits; and, reducing vascular inflammation, since RBEE reduced iNOS involvement and iNOS and TNF-α expression.

- Regarding to the effects of 1% and 5% RBEE-enriched diet over microvessels, an attenuation of the mesenteric resistance arteries alterations in obese Zucker rats was observed by the treatment, through a decreased production of ROS, an increased production and releasing of nitric oxide, and a restoration of calcium-activated potassium channels, protecting the role developed by EDHF in the vasodilatation.

- The extract administration was able to attenuate structural changes of white adipose tissue related to obesity in obese Zucker rats as well as in DIO mice. In the case of the genetic model of Zucker rats, both RBEE concentrations, 1% and 5%, significantly reduced the over-expression of inflammatory mediators such as TNF-α, IL-6, IL-1β and iNOS in the abdominal adipose tissue, and partially in the epididymal fat. In DIO mice, 1% RBEE acted as a modulator of the inflammatory response associated to Metabolic Syndrome, modifying cytokines production and macrophages polarization.

- Globally, obtained results suggest that an EESA-supplemented diet exerts beneficial effects against to metabolic syndrome and its associated cardiovascular and metabolic complications. Therefore, we could consider RBEE as a novel alternative in the functional foods context, since its supplementation could be used as a coadjuvant in the prophylaxis of obesity-related complications and as a dietetic complement in the treatment of Metabolic Syndrome.