ICLIAD 63 (4), 323-456, 2022

p-ISSN 0535-5133
e-ISSN 2477-9393

Volumen 63
No. 4
Diciembre 2022

Investigación Clínica

Universidad del Zulia
Facultad de Medicina
Instituto de Investigaciones Clínicas
"Dr. Américo Negrette"
Maracaibo, Venezuela

Investigación Clínica

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Revista arbitrada dedicada a estudios humanos, animales y de laboratorio relacionados con la investigación clínica y asuntos conexos.

La Revista es de Acceso Abierto, publicada trimestralmente por el Instituto de Investigaciones Clínicas "Dr. Américo Negrette", de la Facultad de Medicina, de la Universidad del Zulia, Maracaibo, Venezuela.

Investigación Clínica está indizada en Science Citation Index Expanded (USA), Excerpta Medica/EMBASE y Scopus (Holanda), Tropical Diseases Bulletin y Global Health (UK), Biblioteca Regional de Medicina/BIREME (Brasil), Ulrich's Periodicals, Journal Citation Reports (USA), Index Copernicus (Polonia), SIIEC Data Bases, Sección Iberoamérica (Argentina) e Infobase Index (India), Redalyc y las bases de datos: SciELO (www.Scielo.org.ve), Revencyt, LILACS, LIVECS, PERIODICA y web de LUZ: http://www.produccioncientificaluz.org/revistas

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Universidad del Zulia
Publicación auspiciada por el
Vicerrectorado Académico
Serbiluz-CONDES

CODEN: ICLIAD
Versión impresa ISSN: 0535-5133
Depósito legal pp 196002ZU37
Versión electrónica ISSN: 2477-9393
Depósito legal ppi 201502ZU4667

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Invest Clin 63(4): 323 - 325, 2022 https://doi.org/10.54817/IC.v63n4a00

EDITORIAL
Leer *versus* citar un trabajo. Análisis de nuestras publicaciones en los últimos años.

El propósito al escribir este Editorial fue enfocar un dilema que enfrentan las publicaciones científicas desde el punto de vista de los autores y de los editores: cuánto vale la citación de un trabajo y cuánto peso se le debe dar a su lectura.

Comenzaremos por hacer un recuento de la actividad de Investigación Clínica en el año 2022. Tanto en el 2021, como en el presente año, se registró un aumento considerable en el número de trabajos recibidos para su posible publicación en "Investigación Clínica". Esto se tradujo en una elevación de las contribuciones publicadas en este año 2022, sobre todo provenientes del extranjero, los cuales alcanzaron el 75% del total de los trabajos publicados. El continente más representado fue Asia, que aportó el 50% de dichos trabajos.

Se implementaron modificaciones editoriales y se incluyeron varias secciones por recomendación de los índices internacionales, las cuales fueron descritas en el último número del 2021. Seguimos contando con una participación activa de árbitros venezolanos, no solo residentes, sino de aquellos que actualmente trabajan fuera del país (EEUU, Ecuador, Méjico, Nicaragua, Argentina, Chile, España y República Checa), que forman parte de la diáspora venezolana. A ellos, así como a todos los demás árbitros que nos han prestado su apoyo, nuestro agradecimiento por la colaboración generosa y desinteresada.

Ahora bien, vamos a analizar la importancia de la lectura de un trabajo publicado en la Revista en comparación con su citación en otro trabajo científico publicado, tema sobre el cual existe una controversia interesante de estudiar.

Gracias a nuestra inserción en el "Crossref", hemos podido conocer el impacto comunicacional de los trabajos publicados, una medida de la extensión de la lectura de dichos trabajos. Las cifras arrojaron que los trabajos publicados en Investigación Clínica se leyeron en un promedio de 1050 veces al mes en los últimos 10 meses; estando en el tope de la búsqueda, como era de esperarse por la época, los referentes a la epidemia de COVID-19 y notándose una preferencia por los artículos de Revisión, lo cual es lo más frecuente, ya que esta forma de publicación es muy utilizada, especialmente por los tesistas.

Otra medida de la consulta a nuestras publicaciones, ha sido la obtenida a partir de la métrica de nuestra página web, la cual reveló que en el año 2021 nos visitaron 1.300 usuarios de 56 países; los usuarios más frecuentes con más de 400 sesiones, fueron de Ecuador, Venezuela y EEUU, seguidos de México e India, con 200 sesiones.

Por otro lado, nuestra inserción en los índices internacionales, especialmente "Web of Science" y "Scopus" ha revelado que el impacto, medido a través de citaciones, no ha sido muy elevado, aunque los resultados difieren en forma importante, al obtenerse cifras muy superiores en el "Web of Science". Es en este punto donde queremos hacer referencia a artículos críticos sobre el impacto de las citaciones, como el de Hirsch [1] de la Universidad de California-San Diego, EEUU, quien comenta el hecho de que las revistas emplean varios métodos para incrementar

su Índice de Impacto; una forma de incrementarlo dice textualmente es "publicando sobre todo artículos de Revisión, los cuales, son más citados que los Trabajos Originales, y el Editor puede forzar al autor a utilizar algunas auto-citaciones espurias para aceptar dicho artículo o no aceptar publicar artículos que se citen poco como los Reportes de Caso". En Investigación Clínica no aplicamos estas tácticas y prácticamente en todos los números predominan Trabajos Originales y se aceptan Reportes de Casos.

Otra forma de conocer el impacto de una revista es el índice "H", usado comúnmente para la medida de la productividad de los investigadores. Según Scopus, nuestra revista ha mantenido un razonable índice H de 22. En relación a este y otros índices, un trabajo de Kuan-Teh Jeang [2] del Instituto Nacional de Salud de EEUU, se refiere a la "Frecuencia de Citaciones vs Frecuencia de Accesos". En dicho trabajo, el autor habla de la discusión sobre estos índices, los cuales se han establecido como presunción de valor científico, haciéndose la pregunta si no será que esa presunción es incorrecta, y si existen otros valores que deben ser considerados. Coloca como ejemplo otras áreas de comunicación como la edición de libros o grabaciones musicales, en las cuales la citación no es importante pero sí la cantidad de lectores, o de música que se escucha, lo que actualmente se mide a través de las redes sociales, y se pregunta si no debería ser lo mismo en la actividad científica. En su trabajo llega a la conclusión que altos valores de lectura sí producen una mayor frecuencia de citaciones, pero que una alta frecuencia de citaciones no siempre se acompaña de alta frecuencia de lectura. Pudiera ser que las citaciones se concentran en un selecto grupo que trabaja específicamente en el tema objeto del estudio, y puede tener poco interés universal.

Otro ejemplo de apoyo a la importancia de la lectura sobre la citación, lo colocan Shanta y col. [3] cuando, citando un Editorial publicado en la revista Journal of Medical Physics en el 2008 establecen que: "La información suministrada en la revista está dirigida a mejorar la práctica clínica de la Medicina Física, y la real medida del impacto de un trabajo es el número de veces que la información contenida en él es usada por el practicante de la Medicina Física para mejorar la calidad de su tratamiento". Por lo tanto, invitamos a los jueces de las revistas, así como a nuestros autores y lectores a hacer una reflexión sobre estas preguntas.

Por nuestra parte seguiremos esforzándonos por conseguir que los trabajos que publiquemos tengan la calidad suficiente para ser citados, pero que también despierten interés por ser leídos y sus resultados puestos en práctica.

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Accessing vs citation of a scientific paper. Analyzing our publications in the last years

In this editorial, I want to focus on a dilemma: Is the citation of a paper much better than accessing and reading it? What is more important for science in real life? A citation may be relevant, but accessing a journal's article could be crucial if it has some application. Our inclusion in Crossref has permitted us to determine the communicational impact of the published articles as a measure of their reading extension, revealing a monthly mean of 1050 readings this year. As we expected, most of the readings came from COVID-19 issues and review articles. Another metric of our publications is related to the number of visitors to our journal web page: in 2021, we received 1300 visits. On the other hand, our presence in WoS and Scopus has shown a modest impact on citations, which is more evident in WoS. About different papers published on the subject, Hirsh [1] pointed out that the journal's impact increases if the readers pay more attention to review articles instead of primary research articles. The H-index is another metric used to determine the journal's impact based on the number of citations. According to Scopus, our journal has an H-index of 22. Jeang [2] mentions, in his article about the H-Index and the differences between the frequency of citations and accessing, that a higher number of readings can produce an increased number of citations. But conversely, a high number of citations of one article does not imply a high number of readings. Finally, in the work of Shanta et al. [3] they concluded that the accurate measurement of the impact of a paper is the number of times a medical practitioner uses the information contained in that paper to improve the quality of the treatment. For our part, we will advocate striving to ensure that the works we publish have sufficient quality to be cited but also to be presented to arouse interest in its reading and their results put into practice.

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Invest Clin 63(4): 327 - 343, 2022 https://doi.org/10.54817/IC.v63n4a01

The effect of varied exercise intensity on antioxidant function, aortic endothelial function, and serum lipids in rats with non-alcoholic fatty liver disease.

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Keywords: different exercise intensity; NAFLD; aortic endothelial cell function; antioxidant; lipid metabolism.

Abstract. This study aimed to compare the effects of diet and exercise of different intensities on antioxidant function, aortic endothelial cell function and serum lipids in NAFLD (nonalcoholic *fatty liver* disease) rats. Fifty Sprague-Dawley (SD) rats (180-220g) were randomly divided into two experimental groups and fed either a standard rodent chow diet (CON; n=10) or a high-fat diet (HFD; n=40). After 16 weeks, the animals that received the HFD were randomly separated into a high-fat control group (HFC; n=10) or three exercise training groups: HFD and low-intensity exercise (LE; n=10), HFD and moderate-intensity exercise (ME; n=10), and HFD and incremental intensity exercise (IE; n=10). These experimental rats keep sedentary or trained for the next six weeks. A detection kit was used to detect nitric oxide synthase (NOs), nitric oxide (NO), malondialdehyde (MDA) and other markers of aortic oxidative stress. The expression levels of endothelial nitric oxide synthase (e-NOS) and endothelin-1 (ET-1) were detected by immunohistochemistry. TC, TG, and other lipid metabolism parameters were detected by an automatic analyzer. Exercise with different intensities could improve lipid metabolism, enhance antioxidant function, reduce MDA ($P<0.01$), increase NO ($P<0.01$), and improve the expression of e-NOS and ET-1 ($P<0.01$) protein levels in NAFLD rats. Decreased blood lipids were exhibited in all exercise groups. Notably, the moderate-intensity exercise demonstrated more effect on increasing glutathione (GSH) contents ($P<0.01$) and decreased the expression of ET-1 protein levels ($P<0.01$). The results showed that exercise at different intensities improved lipid metabolism and enhanced anti-oxidation function. Moderate exercise could improve the function of aortic endothelial cells.

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El efecto del ejercicio de intensidad variada sobre la función antioxidante, la función endotelial aórtica y los lípidos séricos en ratas con enfermedad del hígado graso no alcohólico.

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Palabras clave: diferente intensidad de ejercicio; NAFLD; función de las células endoteliales aórticas; antioxidante; metabolismo de los lípidos.

Resumen. Este estudio tuvo como objetivo comparar los efectos de la dieta y el ejercicio a diferentes intensidades sobre la función antioxidante, la función de las células endoteliales aórticas y los lípidos séricos en ratas NAFLD (con enfermedad del hígado graso no alcohólico) y alimentados con una dieta estándar para roedores (CON; n = 10) o con una dieta alta en grasas (HFD; n = 40). Después de 16 semanas, los animales que recibieron HFD se separaron aleatoriamente en un grupo de control alto en grasas (HFC; n=10) o tres grupos de entrenamiento físico: HFD y ejercicio de baja intensidad (LE; n=10), HFD y ejercicio de intensidad moderada (ME; n=10), y HFD y ejercicio de intensidad incremental (IE; n=10). Estas ratas experimentales se mantuvieron sedentarias o entrenadas durante las próximas seis semanas. Se utilizó un kit de detección para determinar óxido nítrico sintetasa (NO), óxido nítrico (NO), malondialdehído (MDA) y otros marcadores de estrés oxidativo aórtico. Los niveles de expresión de la óxido nítrico sintetasa endotelial (e-NOS) y endotelina-1 (ET-1) se detectaron mediante inmunohistoquímica. El analizador automático detectó TC, TG y otros parámetros del metabolismo de los lípidos. El ejercicio con diferente intensidad mejoró el metabolismo de los lípidos, mejoró la función antioxidante, redujo la MDA (P <0,01), aumentó el NO (P <0,01) y mejoró la expresión de los niveles de proteína e-NOS y ET-1 (P <0,01) en ratas NAFLD. Se observó una disminución de los lípidos en sangre en todos los grupos de ejercicio. En particular, el ejercicio de intensidad moderada demostró un mayor efecto en el aumento del contenido de glutatión (GSH) (P<0,01) y disminuyó la expresión de los niveles de proteína ET-1 (P<0,01). Los resultados mostraron que el ejercicio a diferentes intensidades mejoró el metabolismo de los lípidos y mejoró función antioxidante. El ejercicio moderado podría mejorar la función de las células endoteliales aórticas.

Received: 09-05-2022 Accepted: 16-07-2022

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the hepatic manifestation of metabolic syndrome, and it is the most prevalent liver disease worldwide[1]. The prevalence of NAFLD is approximately 30% in the United States and Europe, with a similar prevalence has been documented in Asian countries[2]. It encompasses a spectrum ranging from simple steatosis to fatty liver with hepatocellular injury, termed nonalcoholic steatohepatitis (NASH), fibrosis and cirrhosis[3].

Moreover, the majority of deaths among NAFLD patients are not only associated with liver-related morbidity and mortality but also related to cardiovascular and other complications. A large number of studies have shown that high-fat diets (HFD) can cause lipid metabolism disturbances, abnormal lipid accumulation, obesity, and NAFLD [4-6]. Free fatty acids (FFA) can cause oxidative stress, which is a primary cause of intravascular dysfunction. Therefore, long-term HFD can inhibit nitric oxide synthase expression in vascular endothelial cells; reduce nitric oxide (NO) production, resulting in abnormal blood vessel endothelial cell function and vascular endothelial dysfunction. NO is produced via NO synthases, which are a family of enzymes catalyzing the production of NO from L-Arginine. For this work, we will consider total nitric oxide synthase (T-NOS), endothelial nitric oxide synthase (e-NOS) and inducible nitric oxide synthase (i-NOS). T-NOS, as the name suggests, is the aggregate nitric oxide synthase (NOS) circulating at any time. At the same time, e-NOS is the endothelial NOS generated in blood vessels and is involved in the regulation of vascular function. i-NOS is inducible NOS which is usually raised in an oxidative environment. As NO expression is altered with endothelial dysfunction, which in turn is associated with NAFLD, finding an effective management solution is, therefore, a current research priority.

Previous experiments have shown aerobic exercise can improve lipid metabolism, oxidative stress [7, 8] and vascular endothelial function [9]. Several pharmacological and non-pharmacological strategies have been proposed to relieve NAFLD-associated deleterious alterations [10]. Among non-pharmacological approaches, physical exercise-mediated multi-systemic adaptations can promote crosstalk between organs and orchestrate pro-metabolic effects known to mitigate metabolism-related disorders such as NAFLD [11]. Keating *et al.* examined that the efficacy of commonly prescribed exercise dose and intensity for reducing liver fat and visceral adipose tissue in an animal experimental model of NAFLD, but no significant differences were found between the dose or intensity of the exercise regimen and reductions in liver fat or visceral adipose tissue [12]. Paradoxically, it has been shown that vigorous and moderate exercise were equally effective in reducing intrahepatic triglyceride (TG) content, but body weight, body fat, waist circumference, and blood pressure with vigorous-moderate intensity exercise were lower than the moderate-intensity group [13, 14]. Similarly, Tsunoda *et al*. showed that vigorous intensity was more effective than moderate-low intensity exercise and moderate-high intensity protocols in preventing NAFLD from progressing to NASH [14]. Two systematic reviews of published studies of NAFLD patients participating in aerobic exercise programs showed that liver fat was significantly reduced. Still, the optimal exercise intensity is undetermined [15, 16], although a growing number of prospective data shows the effects ofdifferent types of exercise on NAFLD[17]. Collectively, these previous findings suggest that the intensity of exercise, rather than the volume or duration, may play a critical role in magnifying the protective effects against NAFLD [18].

The relationship between NAFLD and aortic endothelial function is poorly understood. It is unclear whether exercise could affect aortic endothelial func-

tion in a dietary-induced rat model of NAFLD. Moreover, different exercise intensities may produce varying effects on endothelial function in rat NAFLD models. Therefore, this study compared the impact of different exercise intensities on markers of aortic endothelial function in a HFD-induced NAFLD rat model.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley (SD) rats (180-220g) were purchased from the Guangdong Medical Laboratory Animal Centre (GDMLAC) (Guangzhou, China). According to the principles of the Helsinki declaration, this experiment was approved by the animal experiment ethics checklist of South China Normal University (iacuu-2008-0020). Rats were raised in a specific pathogen free (SPF) facilities environment (23 ± 1°C, humidity 60-70%, 12h light/dark cycle), in the Laboratory Animal Center. SD rats (n=50) were randomly divided into two experimental groups, a control group fed standard rodent chow diet (CON; n=10), and a high-fat diet group (HFD; n=40). After 16 weeks, animals that received the HFD were randomly separated into a sedentary control high fat group (HFC; n=10) or three exercise training groups: HFD and low-intensity exercise (LE; n = 10), HFD and moderate-intensity exercise (ME; n = 10), HFD and incremental intensity exercise (IE; n =10). For the next six weeks, the CON group received ad libitum feeding with standard rodent chow diet and remained sedentary, the HFD group received ad libitum feeding with a high-fat diet and remained sedentary. The LE, ME and IE groups, were fed with high a fat diet and were trained with different exercise training intensities. At the end of six weeks of treatment, the rats were sacrificed after overnight fasting. Blood samples, aorta samples, and liver samples were harvested for analysis. All procedures were performed following the "Guidelines for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH Publication No. 85-23, revised in 1996).

Composition of the HFD

The HFD contained 5% sucrose, 18% lard, 15% egg yolk powder, 0.5% sodium cholate, and 1% cholesterol, added to the 60.5% basic standard rodent chow diet.

Exercise experimental protocol

All animals were familiarized with treadmill running (DSPT202, Qianjiang Technology Company, Hangzhou, China) at 0-15 m/min, 10-20 min per day, for six consecutive days. An electrified grid (0.6–mA intensity) was placed behind the belt of the treadmill to induce running. The rats that failed to run regularly were excluded from the training protocol. The exercise program involved 60 min/day, five days per week, for a total of six weeks. The rats performed exercises based on a protocol described previously[19-21], with some modifications. The daily training intensity program was for each group respectively: LE group: 15 m/min, ME group: 20 m/min, and IE group consisted of running 10 minutes at 15 m/min, followed by a gradual increase in intensity at 20 m/min for 30 min, and increase in intensity to 27 m/min for 20 min on a motor–driven treadmill.

Outcome Measures

The primary outcome measures were the markers of oxidative stress in the aorta; T-NOS and i-NOS. superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT), glutathione (GSH) and Total antioxidant capacity (T–AOC).

The secondary outcome measures were; the presence of aortic endothelin-

1(ET-1) and e-NOS, body mass, liver mass and lipids.

We also confirmed the existence of NAFLD (liver histology) by hematoxylin and eosin (H&E) staining of embedded liver tissue samples.

Lipids

Blood samples were collected from the abdominal aorta and centrifuged at 3000 rpm for 15 min, and then serum was collected. The serum TG levels (mmol/l), total cholesterol (TC) levels (mmol/l), low–density lipoprotein cholesterol (LDL–c) levels (mmol/l), high-density lipoprotein cholesterol (HDL–c) levels (mmol/l), were detected with an automatic analyser (Toshiba AcuteTBA-40FR, Toshiba Corporation, Tokyo, Japan).

Markers of aortic oxidative stress

The aorta was removed to an ice plate, then placed in liquid nitrogen and the sample was saved for testing. Prepared fresh aorta samples were ground in saline solution to make 10% aorta homogenates, followed by centrifugation for 20 min at 4°C. The resulting supernatant was collected using specific kits according to the manufacturer's instructions. NOS (T-NOS and i-NOS) were detected using an assay kit (Colorimetric method), (Nanjing Jiancheng Corp., Nanjing, China), NO assay kit (Nitrate reductase method).

MDA contents in the aorta were quantified using a lipid peroxidation MDA assay kit (TBA method) (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's protocol. CAT activity assay kit (Visible light method), SOD, T–AOC activity and GSH contents were determined using a reagent kit (Colorimetric method), (Nanjing Jiancheng Corp., Nanjing, China).

Endothelin-1 and Nitric oxide synthase

ET-1 and e-NOS were measured by immune-histochemical analysis. After the abdominal cavity was opened, the aorta was wholly and quickly separated; the fixed aorta was embedded in paraffin, sliced into 5-μm-thick sections, and mounted on glass slides. The immunohistochemistry was performed with a PowerVision two-step immunohistochemistry detection kit. ET-1 and e-NOS antibodies were obtained from (Bioss Biotechnology Co., Ltd. Beijing, China). Samples were observed through JVC3-CCD camera (Nikon Corp., Tokyo, Japan), and Image-Pro Plus image (Media Cybernetics Corp., USA) processing software system was used for image acquisition and analysis. The brown granules visible in the cytoplasm or nucleus were considered the positive expression of aortic endothelial cells. The number of positive cells per section were counted in 10 random fields (400x magnification), and the percentage of positive cells (positive cells/total cells × 100%) was calculated. Three non-consecutive sections were selected from each specimen, and those indices were averaged.

Characterization of non-alcoholic fatty liver disease

To characterize NAFLD, rat livers were fixed with 10% formalin, and the paraffin-embedded liver tissue samples were cut into 10μm sections for H&E staining. At least three randomly selected liver section images were then captured digitally (400x magnification), and each set of images was examined and photographed using nikon Eclipse Ci light microscope (Nikon Tokyo, Japan).

Statistical analysis

The statistical package for the social sciences (SPSS version 20.0, IBM Corp., USA) software was used for one-way ANOVA and Tukey's significant difference post

analysis. The Graph Pad Prism (version 5.0; Graph Pad Prism Software, La Jolla, CA, USA) software was used to draw the chart. The statistical results were expressed as means ± standard deviations (M ± SD). P value of ≤ 0.05 denoted a statistically significant difference.

RESULTS

Effect of different exercise training intensities on lipid metabolism disorders and liver histology

As shown in Fig. 1, liver histology was evaluated by H&E staining. CON group rats tissue exhibited well-arranged hepatic cords, cells with round and central nuclei, a lobular structure and an array of wheel–shaped cells along the centrilobular vein. However, in the HFC group, lipid droplets were observed in the liver sections (Fig. 1B). Lipid droplet volumes and quantities were reduced with different exercise intensities (Figs. 1C, 1D, and 1E).

As shown in Table 1, the serum TC, TG, LDL-c and FFA were lowest in the CON group, but TC, TG and LDL-c were significantly decreased in the LE, ME and IE groups compared with the HFC group. No difference in serum HDL-c was observed between groups. Notably, TG, TC, and LDL were not significantly different between the three exercise groups.

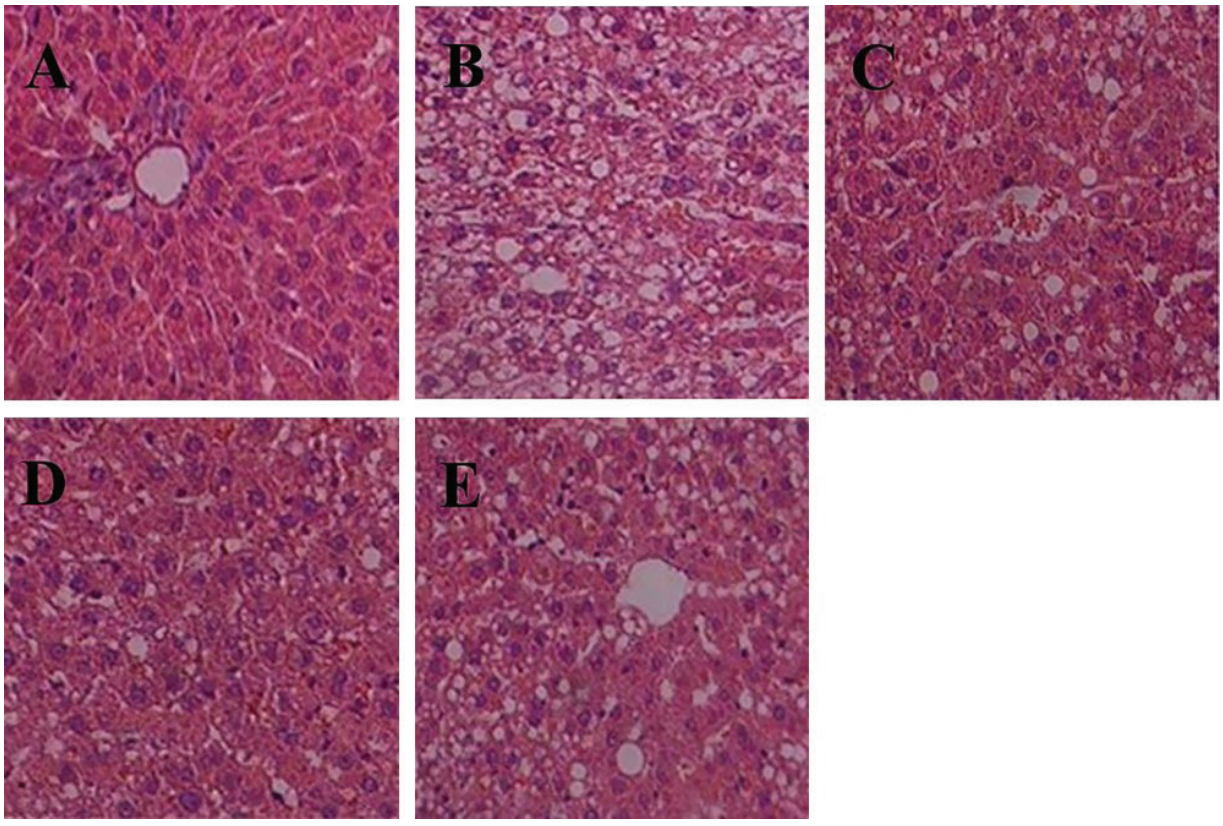

Fig. 1. The optical microscope image of H&E staining in Rat Liver Tissue (400×).
CON= control group (A); HFC= high fat control group (B); LE= HF and low intensity exercise (C); ME= HF and middle intensity exercise (D); IE= HF and incremental intensity exercise (E).

Table 1
The change of blood lipids in each group.

Groups	TG (mmol/L)	TC (mmol/L)	LDL–c (mmol/L)	HDL–c (mmol/L)	FFA (umol/L)
CON	0.45±0.13	1.36±0.33	0.26 ± 0.06	0.41 ± 0.01	313.1 ± 23.1
HFC	1.55±0.21**	5.09±0.24**	1.88 ± 0.13**	0.32 ± 0.07	627.2 ± 97.8**
LE	0.87±0.30##**	4.17±0.20##**	1.13± 0.11##**	0.35 ± 0.11	569.2 ± 39.4**
ME	0.80±0.20##**	4.06±0.18##**	1.06± 0.17##**	0.37 ± 0.08	558.2 ± 68.1**
IE	0.79±0.18##**	4.12± 0.16##**	1.05± 0.16##**	0.37 ± 0.09	567.6 ± 49.7**

**, $P < 0.01$ compared with CON group; ##, $P < 0.01$ compared with HFC group.
All data are expressed as mean ± SD; 8–10 animals per group were used. CON= control group; HFC= high fat control group; LE= HFD and low intensity exercise; ME=HFD and middle intensity exercise; IE= HFD and incremental intensity exercise.

Effect of different exercise training intensities on body mass and liver mass

As shown in Fig. 2, after six weeks of treatment, body mass of the rats in each group were not significantly different (Fig. 2A). Liver mass were lower in the ME group than in the HFC group ($P<0.05$), otherwise there was no significant difference among the exercise groups (Fig. 2B).

Effect of different exercise training intensities on aortic endothelial cell oxidative stress

As shown in Fig. 3, compared with the CON group, CAT, GSH, and T–AOC showed a significant reduction in the HFC group ($P<0.01$; Fig 3C, 3D and 3E). After six weeks of exercise training, SOD, CAT, and T–AOC in LE, ME, and IE group were significantly higher compared to

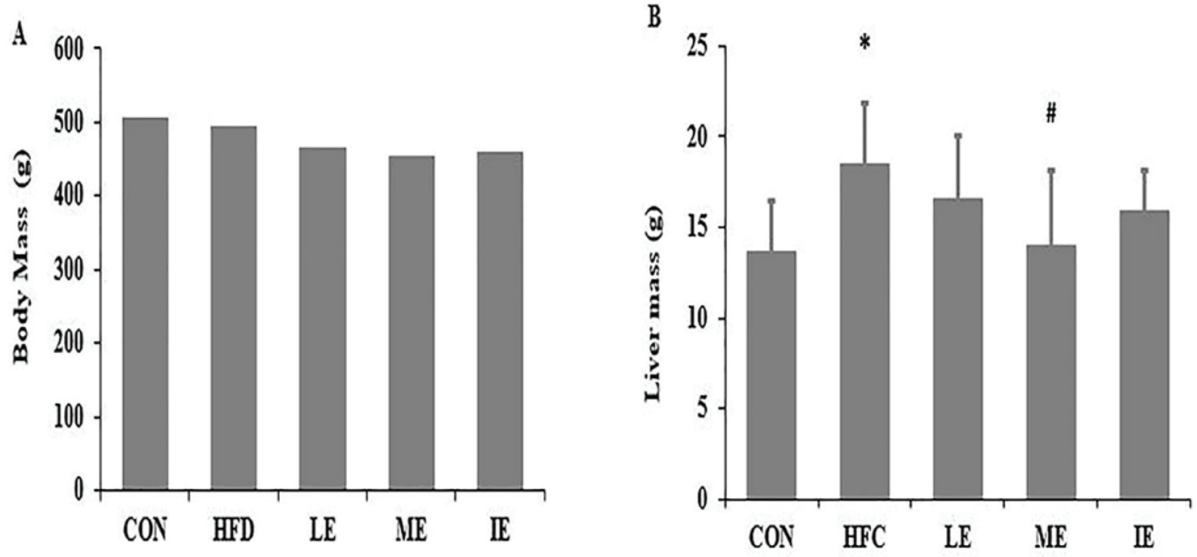

Fig. 2. Effects of exercise training on body mass and liver mass.
Body mass (A), liver mass (B) of each group. *, P< 0.05 compared with CON group; #, P < 0.05 compared with HFC group;
CON= control group; HFC= high fat control group; LE= HF and low intensity exercise; ME= HF and middle intensity exercise; IE= HF and incremental intensity exercise.

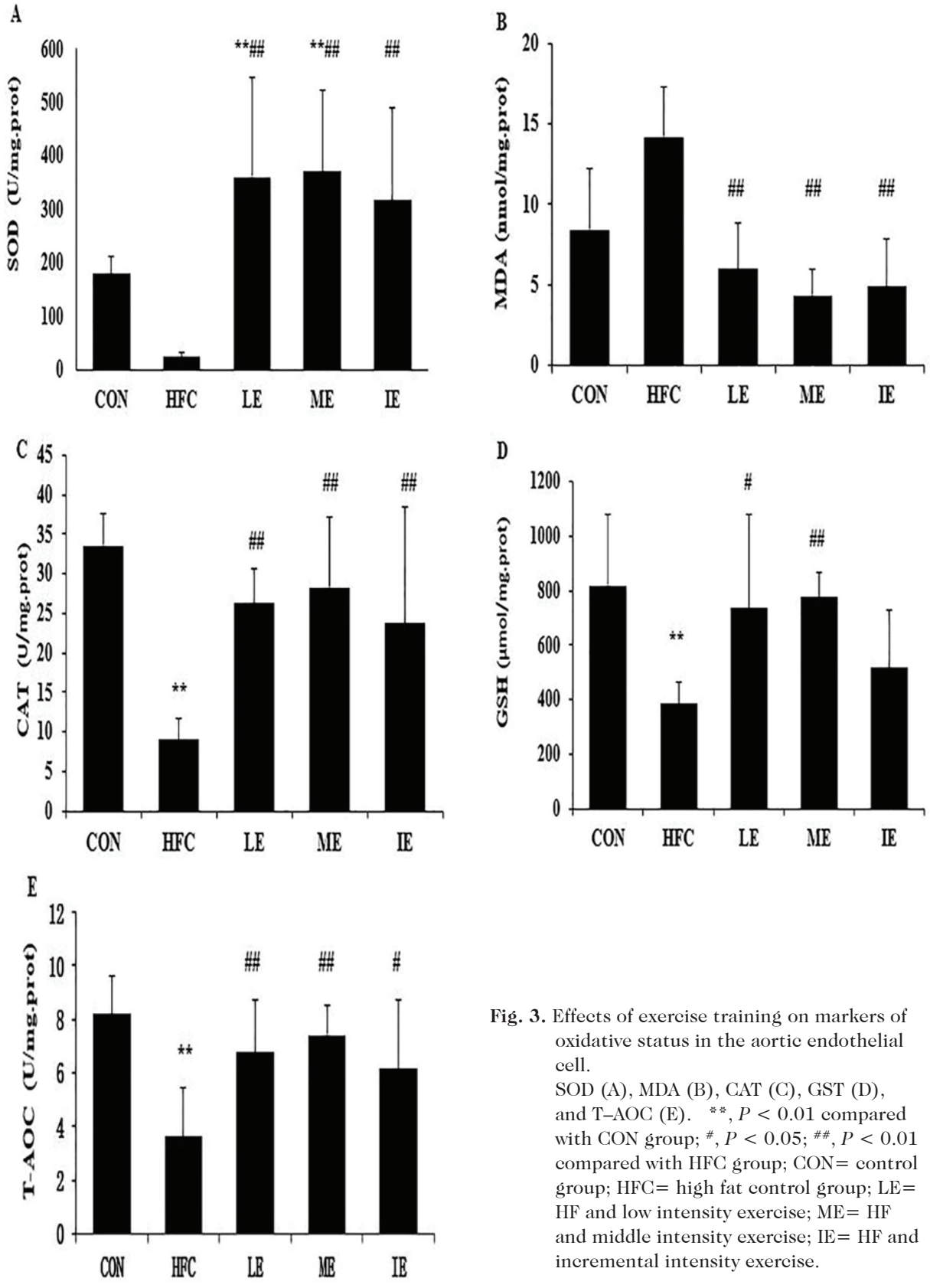

Fig. 3. Effects of exercise training on markers of oxidative status in the aortic endothelial cell.
SOD (A), MDA (B), CAT (C), GST (D), and T–AOC (E). **, $P < 0.01$ compared with CON group; #, $P < 0.05$; ##, $P < 0.01$ compared with HFC group; CON= control group; HFC= high fat control group; LE= HF and low intensity exercise; ME= HF and middle intensity exercise; IE= HF and incremental intensity exercise.

the HFC group (Fig. 3A, 3C and 3E), whereas the MDA levels were decreased compared to the HFC group ($P<0.01$; Fig. 3B). In addition, LE and ME groups exhibited significantly increased GSH compared to the HFC group ($P<0.05$ and $P<0.01$ respectively; Fig. 3D).

Effect of exercise training intensity on aortic NOS and NO activities

Table 2 shows that aortic T-NOS activity was higher in the CON versus HFC ($P<0.01$) and IE ($P<0.05$) groups; however, only the low intensity (LE) group showed a significant elevation compared to HFC ($P<0.05$). i-NOS activity was higher in the HFC versus CON group ($P<0.01$); the LE and ME groups showed a significant reduction compared to HFC ($P<0.01$), while the LE and ME groups showed a significant reduction compared to the IE group ($P<0.01$).

Table 3 shows that the NO content in the aorta was higher in the CON versus HFC ($P<0.01$) and all exercise ($P<0.01$) groups, all exercise groups showed a significant elevation compared to HFC ($P<0.01$).

Effect of different exercise training intensities on e-NOS and ET-1 expression in the aorta

The expression of e-NOS protein levels in the aorta was significantly lower in the HFC group than in CON group ($P<0.05$), whereas there was no difference between the three exercise groups (Table 4 and Fig. 4). The expression of ET-1 protein levels was significantly higher in the HFC, LE, and IE groups compared to the CON group. The ET-1 levels were significantly decreased by moderate-intensity exercise training ($P<0.01$) (Table 5 and Fig. 5).

DISCUSSION

The aim of our study was to investigate the effects of varying exercise in-

Table 2
The change of aortic nitric oxide synthase (NOS) activity in each group (U/mg prot).

	CON	HFC	LE	ME	IE
T-NOS	6.04 ± 0.32	3.09 ± 0.31**	4.79 ± 0.25#	4.19 ± 0.31	4.17 ± 0.19*
iNOS	1.29 ± 0.14	2.85 ± 0.27**	2.03 ± 0.17**##&&	2.18 ± 0.23**##&&	2.63 ± 0.16**

T-NOS, Total Nitric Oxide Synthase; iNOS, inducible nitric oxide synthase; *, $P<0.05$; **, $P<0.01$ vs CON; #, $P<0.05$; ##, $P < 0.01$ vs HFC; &&, $P < 0.01$ vs IE.
All data are expressed as mean ± SD; 8–10 animals per group were used. CON= control group; HFC= high fat control group; LE= HFD and low intensity exercise; ME=HFD and middle intensity exercise; IE= HFD and incremental intensity exercise.

Table 3
The change of aortic NO in each group (U/mg prot).

	CON	HFC	LE	ME	IE
NO	202.34±11.46	150.21±7.72**	176.61±9.07**##	183.18±8.10**##	175.43±7.02**##

NO, Nitric Oxide ;*, $P < 0.05$; **, $P < 0.01$ vs CON; #, $P < 0.05$ vs HFC.
All data are expressed as mean ± SD; 8–10 animals per group were used. CON= control group; HFC= high fat control group; LE= HFD and low intensity exercise; ME=HFD and middle intensity exercise; IE= HFD and incremental intensity exercise.

Table 4
The change of e-NOS expression in each group.

	CON	HFC	LE	ME	IE
eNOS	0.52±0.13	0.35±0.08*	0.43±0.02	0.41±0.07	0.38±0.21

eNOS, Endothelial nitric oxide synthase; *, $P < 0.05$; vs CON.
All data are expressed as mean ± SD; 8–10 animals per group were used. CON= control group; HFC= high fat control group; LE= HFD and low intensity exercise; ME=HFD and middle intensity exercise; IE= HFD and incremental intensity exercise.

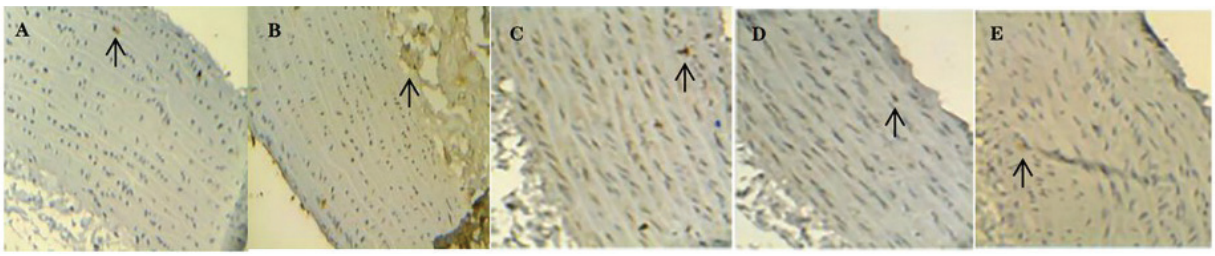

Fig. 4. Effects of exercise training on aortic e-NOS expression.
CON= control group (A); HFC= high fat control group (B); LE= HF and low intensity exercise (C); ME= HF and middle intensity exercise (D); IE= HF and incremental intensity exercise (E).

Table 5
The change of ET-1 expression in each group.

	CON	HFC	LE	ME	IE
ET-1	0.25 ± 0.03	0.39 ± 0.04**	0.33 ± 0.05*	0.29 ± 0.02##	0.35 ± 0.09**

ET-1, Endothelin-1; *, $P < 0.05$; **, $P< 0.01$ vs CON; ##, $P < 0.01$ vs HFC.
All data are expressed as mean ± SD; 8–10 animals per group were used. CON= control group; HFC= high fat control group; LE= HFD and low intensity exercise; ME=HFD and middle intensity exercise; IE= HFD and incremental intensity exercise.

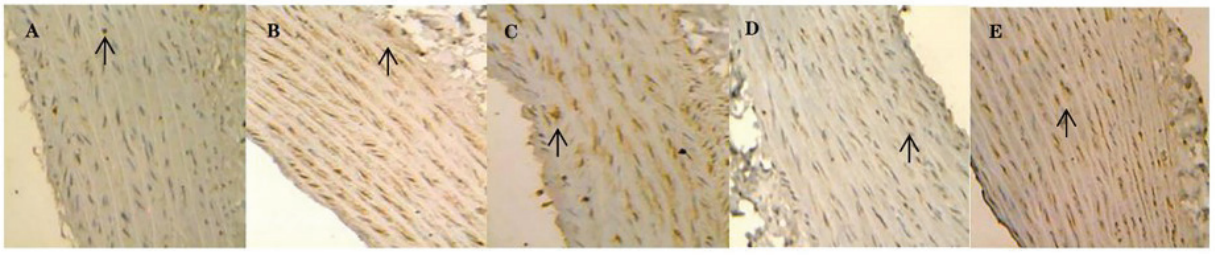

Fig. 5. Effects of exercise training on aortic ET-1 expression.
CON= control group (A); HFC= high fat control group (B); LE= HF and low intensity exercise (C); ME= HF and middle intensity exercise (D); IE= HF and incremental intensity exercise (E).

tensities on aortic endothelial function in high fat diet-induced NAFLD rats. The specific process is shown in Fig 6. We can confirm that HFD induced vascular endothelial dysfunction in NAFLD rats. We found that exercise enhanced anti-oxidation function and improved some markers of aortic endothelial cell function. T-NOS activity appeared to respond best to low intensity exercise. i-NOS activity was lower only in the LE and ME groups. Moderate intensity exercise demonstrat-

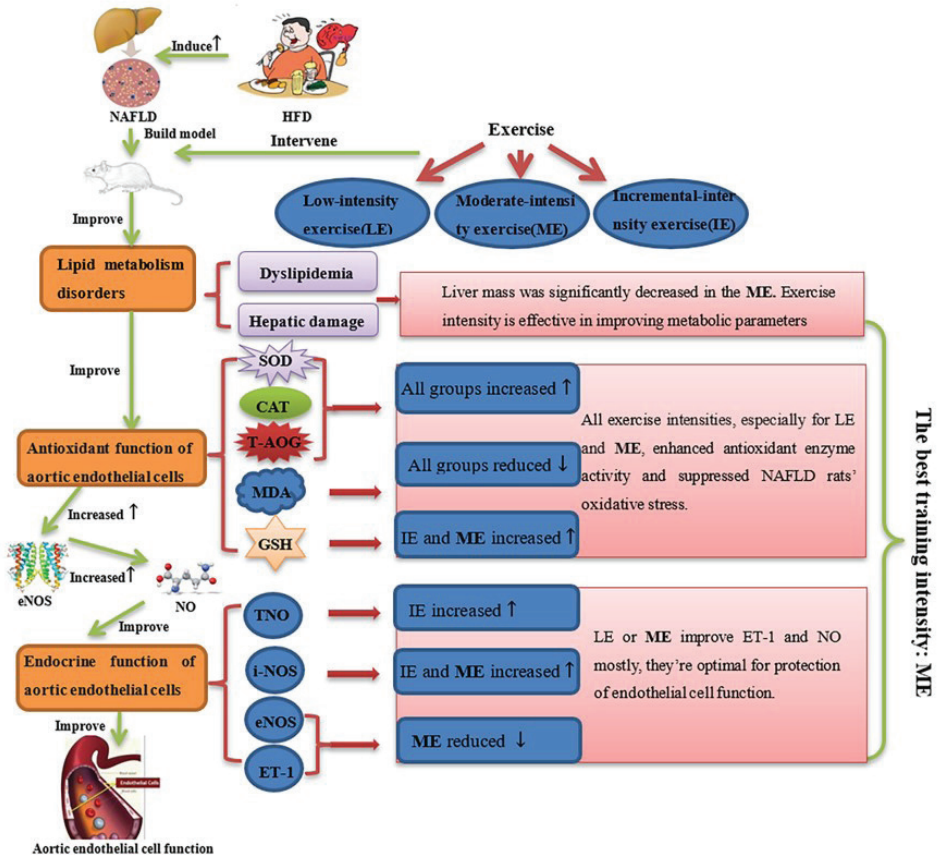

Fig. 6. Effects of different exercise intensities on antioxidant function aortic endothelial cell function and blood lipid in rats with non-alcoholic fatty liver disease.

ed the greatest effect on decreasing the expression of the potent vasoconstrictor ET-1 levels and the expression of NO, whereas GSH was raised in this group. Decreased blood lipids were exhibited in all exercise groups.

The impact of different exercise training intensities on lipid metabolism disorders

We demonstrated that three different exercise training intensities were equally effective in alleviating dyslipidaemia as well as hepatic damage in a diet-induced rat NAFLD model. These results suggested that the therapeutic effect of exercise training in dyslipidaemia and hepatic injury is unrelated to exercise intensity. This notion is supported by meta-analytic work [22]. Moreover, our study did not find any improvement in body mass or HDL-c in any group. However, it should be noted that liver mass was significantly decreased in the moderate-intensity group.

Exercise plays a vital role in improving lipid metabolism disorders and is increasingly seen as adjunctive therapy for the prevention and treatment of NAFLD[16, 17, 23]. Machado *et al.* showed that exercise intensity would be more effective in improving metabolic parameters than frequency or duration [24]. A retrospective study indicated that moderate and vigorous-intensity physical activity yielded similar health benefits to low, in terms of the measured body adiposity and serum TG [25]. Two stud-

ies showed that vigorous-intensity interval training and continuous moderate-intensity exercise have the same effect on lowering the serum FFAs, TG of NAFLD in animals [17, 26]. Suk *et al*. also indicated that high-intensity exercise improved lipid metabolism in the liver of rats[27]. Fisher *et al.* also did not find any improvements in body weights and HDL-c between groups of differing exercise intensities[28]. However, Khammassi *et al*. showed that high-intensity interval training may be particularly useful in overweight/obese youth to improve body composition and lipid profile [29].

The impact of different exercise training intensities on the antioxidant function of aortic endothelial cells

Our study showed that CAT, GSH, and T–AOC was significantly reduced in the HFC group, compared with the CON group, which confirmed the expected effect of HFD induced NAFLD rat model. After six weeks of exercise training SOD, CAT, and T–AOC were significantly increased, conversely, MDA was reduced considerably in all exercise groups. Furthermore, low intensity and moderate-intensity exercise increased GSH.

Previous work has shown that HFD increase lipid peroxidation and destroy the balance of the oxidative and antioxidative systems[30]. Moreover, oxidative stress and increased ROS production are the primary cause of dysfunction in aortic endothelial cells [19, 20]. The relationship between exercise and oxidative stress is extremely complex, depending on the mode, intensity, and duration of the exercise. Pingitore *et al*. noted that regular moderate training in humans appears beneficial for oxidative stress and health. Conversely, acute exercise leads to increased oxidative stress[21], presumably as their period of adaptation that is missing from acute exercise training. Pereira *et al.* also showed high-intensity exercise might induce oxidative stress[31]. Li *et al*. reported that SOD activity and GSH were significantly raised after rats were exercised at medium intensity [32]. Radak *et al*. also indicated that moderate exercise significantly increased the activity of antioxidant enzymes[33]. However, Lu *et al*. reported that high-intensity exercise was superior to the moderate-intensity in attenuating oxidative stress and improving glucolipid metabolism in post-MI rat myocardium[34]. Jamurtas *et al*. also found high intensity to be superior to moderate intensity for reducing oxidative stress in healthy male humans[35]. It, therefore, remains unclear which exercise intensity is optimal for improving antioxidant function.

In our study, we elucidated that all exercise training intensities, especially for low intensity and moderate intensity, enhanced antioxidant enzyme activity and suppressed NAFLD rats' oxidative stress. We speculated, and our data support the notion that incremental exercise may increase reactive oxygen species (ROS) production during incremental exercise leading to the oxidation of protein, lipids or nucleic acid [36]. The production of ROS during exercise is also accompanied by a reduction of antioxidant capacity [37]. However, our data lack measures of ROS production and other related oxidative stress markers. So further studies of the molecular mechanisms involved in antioxidation may be indicated.

The impact of different exercise training intensities on endocrine function of aortic endothelial cells

Studies have shown that a high-fat diet can lead to lipid abnormalities, vascular endothelial damage, reduced NO con-

tent produced by endothelial cells, resulting in impaired endothelial function [38]. The role of NO in the liver largely depends on the type of NOS that catalyzes its production. e-NOS plays a beneficial role in alcoholic liver disease, while i-NOS plays an important role in alcohol-induced liver damage. In the process of oxidative stress, the production of i-NOS can be induced, and a large amount of NO from i-NOS can aggravate the liver damage caused by oxidative stress, while NO produced from e-NOS can resist the effect of superoxide [39]. The results of this experiment showed that compared with the CON group, the expression of ET-1 ($P<0.01$) in the aorta of the HFC group increased significantly, while the expression of no and e-NOS ($P<0.05$) decreased, and the expression of i-NOS ($P<0.01$) increased, suggesting that long-term high-fat diet will cause oxidative stress in rats and then lead to vascular endothelial dysfunction.

Exercise can effectively improve the function of aortic endothelial cells, but the optimal exercise intensity is still unclear. The study of Hambrecht et al. showed that exercise training, at symptom-limited intensity, improves arterial endothelial cell function in people with heart disease [40]. Several previous studies have shown low and moderate-intensity exercise to have a positive effect on aortic endothelial cell function of rats [41]. Shaodong *et al*. indicated that aerobic exercise, of unknown intensity, decreases the production of lipid oxidation products, and thus prevents damage to endothelial cells in rats with dyslipidemia[42]. Furthermore, a previous experiment demonstrated that moderate-intensity exercise could reduce the expression of ET-1 levels, induced by aortic injury in mice [43]. Wang *et al*. and Archana *et al*. found that moderate-intensity exercise is optimal for raising serum NO [7,44]. However, Morishima *et al*. indicated endothelial function was maintained by conducting high-intensity resistance exercise[45]. Meta-analysis showed that high-intensity training seems to have a superior effect on the improvement of endothelial function compared with moderate exercise in cardiac patients [46].

Our results show that although the reduction of e-NOS expression level in the ME group is not significant compared with the HFC group, it still shows a downward trend and is stronger than the IE group, with the fastest decreasing trend in the LE group. In the results of NO index, compared with the HFC group, the expression levels of NO in different exercise intensity groups were significantly increased ($P<0.01$), but the ME group had the greatest increase. In the experimental results of ET-1, we found that the expression levels of ET-1 in different exercise intensity groups were significantly reduced, and the ME group and IE group showed a very significant difference. At the same time, we found that compared with the IE group, the i-NOS activity of the LE group and the ME group was significantly decreased ($P<0.01$), which may be due to the intensity of the latter can better improve oxidative stress, leading to the reduction of i-NOS. To sum up, these results show that moderate-intensity exercise can make the most significant improvement in endothelin-1 and nitric oxide levels, which means that moderate-intensity exercise is the most ideal for protecting endothelial cell function.

Our experiments show that exercise can reduce the expression of aortic e-NOS and ET-1 protein levels, improved lipid metabolism. However, the role and underlying mechanism of exercise training in NAFLD related aortic endothelial cell function remain poorly understood. Notably, moderate intensity exercise

demonstrated more effect on decreasing the expression of ET-1 protein levels, and GSH. Therefore, we believe that moderate exercise demonstrated improved aortic endothelial cell function, is underlined by the following: (i) Moderate-intensity exercise improves lipid metabolism, promoting fat mobilization and lipid energy catabolism[25]. (ii) Moderate-intensity exercise has a beneficial anti-oxidative effect. (iii) The reduced ET-1 and increased NO expression were significant in the ME group, ultimately improving aortic endothelial cell function[47,48]. We do, however, concede that results of other works are conflicting.

ACKNOWLEDGEMENTS

First and foremost, I would like to show my deepest gratitude to Dr. Neil Smart, a respectable, responsible and resourceful scholar, who has provided me with valuable guidance. His keen and vigorous academic observation enlightens me. I shall extend my thanks to Dr. Li for all his kindness and help.

Funding

This project was supported by Shaanxi Science and Technology Association Project (2021JQ586, 2021JQ834), a research grant from the Special Research Project of the Education Department of Shaanxi Province (19JK0017), Projects of Shaanxi Provincial Bureau of sports(2021037), and Shaanxi Social Science Foundation Project (2020Q017). We would also like to thank Shou-bang Li who assisted in designing the project and performing daily training over the course of the study and also thank Guang-hua Wang, Rong-ping Wu, Zhen-Qing Lv, and Zhan Jin who assisted in improving the quality of the figures. We would also like to thank Yi-ling Ren, Quan-cheng Zhang, Qin Liu and Xian-li Lv for their work in collecting and querying the information.

Conflict of interest

No conflicting financial interests exist.

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Contributions of authors

LR and GW drafted and revised the manuscript; SL assisted in designing the project and performing daily training over the course of the study; GW, RW, Z-QL, and ZJ assisted in improving the quality of the figures; YR, QZ, QL and XL work in collecting and querying the information.

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Invest Clin 63(4): 344 - 352, 2022 https://doi.org/10.54817/IC.v63n4a02

Apurinic/apyrimidinic endonuclease 1 mRNA level in peripheral blood neutrophils is associated with asthma.

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Keywords: apurinic/apyrimidinic endonuclease; asthma: mRNA; inflammation.

Abstract. Apurinic/apyrimidinic endonuclease 1 (APE1) is a multifunctional key protein. Recent studies suggest APE1 is closely associated with inflammatory response, but its role in asthma remains unknown. We recruited 116 patients with asthma, including 50 with severe asthma (NSA) and 66 with non-severe asthma (SA), and 140 controls. Serum APE1 was detected using the ELISA method. APE1 mRNA in peripheral blood neutrophils and eosinophils were detected using real-time PCR assays. Compared to healthy controls, we observed significant elevations of serum APE1 mRNA levels in peripheral neutrophils (~1.75 folds increase, $p<0.05$) and eosinophils (~2.2 folds increase, $p<0.05$) in patients with asthma. The peripheral blood neutrophil APE1 mRNA can distinguish asthmatic patients from healthy controls with the area under the curve (AUC) 0.893 and a 95% confidence interval (CI) 0.847-0.938 ($p < 0.001$). Also the APE1 mRNA can identify severe asthma from non-severe asthma (AUC 0.759, 95% CI, 0.674-0.846; $p < 0.001$). However, The serum APE1 and eosinophil mRNA levels did not correlate with asthma incidence and severity. Our finding confirms the association between APE1 and asthma and suggests that peripheral blood neutrophil APE1 mRNA may be used as a marker for this condition.

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El nivel de ARNm de la endonucleasa 1 apurínica/apirimidínica en los neutrófilos de sangre periférica se asocia con el asma.

Invest Clin 2022; 63 (4): 344 – 352

Palabras clave: apurínica/apirimidínica endonucleasa; asma: mRNA; inflamación.

Resumen. La endonucleasa apurínica/apirimidínica 1 (APE1) es una proteína clave multifuncional. Estudios recientes sugieren que APE1 está estrechamente asociada con la respuesta inflamatoria, pero hasta el momento se desconoce su papel en el asma. Reclutamos a 116 pacientes con asma, incluidos 50 con asma grave (NSA) y 66 con asma no grave (SA), y 140 controles. Se detectó APE1 en suero usando el método ELISA. El ARNm de APE1 en neutrófilos y eosinófilos de sangre periférica se detectó mediante ensayos de PCR en tiempo real. En comparación con los controles sanos, observamos una elevación significativa de los niveles séricos de ARNm de APE1 en pacientes con asma en neutrófilos periféricos (aumento de ~1,75 veces, $p<0,05$) y eosinófilos (aumento de ~2,2 veces, $p<0,05$). El ARNm de APE1 de neutrófilos de sangre periférica puede distinguir a los pacientes asmáticos de los controles sanos con un área bajo la curva (AUC) de 0,893 y un intervalo de confianza (IC) del 95% de 0,847 a 0,938 ($p<0,001$). Además, el ARNm de APE1 puede identificar el asma grave del asma no grave (AUC 0,759, IC del 95%, 0,674-0,846; $p < 0,001$). Sin embargo, el nivel sérico de APE1 y ARNm de eosinófilos no mostró correlación con la incidencia y la gravedad del asma. Nuestro hallazgo confirma la asociación entre APE1 y asma y sugiere que el ARNm de APE1 de neutrófilos en sangre periférica puede usarse como marcador para el asma.

Received: 20-12-2021 Accepted: 13-07-2022

INTRODUCTION

Asthma is a major public health problem worldwide. It is a multifactorial disease characterized by chronic airway inflammation, leading to bronchial hyperresponsiveness and airway remodeling [1]. Neutrophils and eosinophils are two major pro-inflammatory cell types that play essential role in the pathogenesis of asthma [1-3]. So far, few biomarkers have been evaluated to reflect the airway inflammation in the asthmatic patients, but with unsatisfactory sensitivity or reliability.

The apurinic/apyrimidinic endonuclease 1 (APE1) is a multifunctional key protein initially identified to play an important role in the base-excision repair by recognizing the abasic site [4, 5]. Besides its DNA repair function, recent studies showed that APE1 also regulates the expression of different transcription factors, notably, the inflammatory pathway regulator NF-κB, thus contributing to inflammation regulation [6]. It has been proved that APE1 controls IL-6 and IL-8 expression through its redox function [7]. APE1 also regulates inflammatory response in macrophages and keratinocyte [8,9]. Indeed, APE1/Ref-1 has been viewed as an emerging therapeutic target for various inflammatory diseases, including inflammatory pain sensi-

tization, murine myocarditis and spontaneous chronic colitis [10-12].

The association between APE1 and asthma has not been established yet. Based on the established association between APE1 and inflammatory diseases, we hypothesized that APE1 may play a role in asthmatic inflammation. To test this notion, we detected the serum APE1 protein expression levels, mRAN levels from neutrophils and eosinophils isolated from peripheral blood in adult asthmatic patients and healthy controls.

PATIENTS AND METHODS

Study subjects

The diagnosed asthmatic patients and healthy controls were enrolled at the Department of Respiration, Shidong Hospital of Yangpu District between March August, 2018 and October, 2020. The diagnosis of asthma was made in line with the criteria of the Global Initiative for Asthma (GINA) and described elsewhere [13]. The asthmatic subjects were classified as patients with severe asthma (SA) and patients with non-severe asthma (NSA) by the International European Respiratory Society/American Thoracic Society guidelines [14]. Any patient who had known to have underlying respiratory diseases other than asthma was excluded. We also recruited sex and age matched healthy individuals who had annual checkups at our hospital, but did not have any acute or chronic illness (such as cancer, inflammatory diseases, cardiovascular diseases, etc.), atopic diseases or any symptoms of obstructive airway disease. The body mass index (BMI), smoking status, asthma duration (years), allergic history, blood eosinophils and blood neutrophils counts were obtained from their medical charts.

Ethical statement

The ethical committee of Shidong Hospital of Yangpu District approved the study. This research was conducted in accordance with the principles embodied in the Declaration of Helsinki. All participants were given written informed consent forms to participate in the study.

Pulmonary function

Pulmonary function tests were performed using a SYSTEM 21® device (Minato Medical Science Co., Osaka, Japan), according to the criteria of the American Thoracic Society (ATS)/European Respiratory Society and the Japanese Respiratory Society [15]. The pulmonary function was measured and included the percentage of predicted volume (FEV1% pred).

Serum samples collection and protein quantification

Peripheral blood was drawn in each participant, followed by centrifugation at 3500 rpm for 10 min to isolate serum. Serum samples were collected and APE1 levels were determined using Human APEX1 ELISA kit (Cusabio, Houston, USA). The optical density (OD) was detected with an EnSpire microplate reader (PerkinElmer, Waltham, USA), at a wavelength of 450 nm with a correction set at 540 nm. The concentration of serum APE1 (pg/mL) was calculated using the standard curve. The serum high-sensitivity C-reactive protein (Hs-CRP) was detected using human high sensitivity C-Reactive Protein ELISA kit (Sunlong Biotech, Hangzhou, China) according the manufacturer's protocol. The total IgE level was detected using Human IgE ELISA Kit (Abcam Biotech, Waltham, MA, USA) according the manufacturer's protocol.

RNA isolation and reverse transcription and real-time PCR

Neutrophils and eosinophils were isolated from fresh drawn peripheral blood using the MACSxpress Whole Blood Eosinophil Isolation Kit and MACSxpress Whole Blood Neutrophil Isolation Kit (Miltenyi Biotec Inc., Bergisch Gladbach, Germany), according to the manufacturer's manual. Total RNA was extracted using the TaKaRa RNA PCR

Kit (Takara, Dalian, China) from neutrophils and eosinophils. The expression of APE1 mRNA was performed by quantitative real-time polymerase chain reaction (rt-qPCR) with the SYBR Premix Ex Taq II (Takara, Dalian, China). All samples were performed in triplicate. β-actin was applied for the internal normalization of RNA. The PCR reaction was performed at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 61°C for 30 s. The comparative Ct method (ΔCt) was exploited to calculate the relative expression levels of miRs. The mean cycle threshold (Ct) values and deviations between the duplicates were calculated for all samples. The primers for the APE1 were as following: miR-30,Forward: CTGCTCTTGGAATGTGGATGGG, Reverse TCCAGGCAGCTCCTGAAGTTCA. β-actin, Forward AGAGCTACGAGCTGCCTGAC and reverse GGATGCCACAGGACTCCA.

Statistical analysis

The data are expressed in terms of mean (± standard deviation). Student's t-test and one-way ANOVA were used to compare two or more groups. Pearson's correlation analysis was conducted and the correlation coefficients (r2) were used to measure correlation. A receiver operating characteristic (ROC) curve was performed to the diagnostic value of serum APE1 and its mRNA in neutrophils and eosinophils in the discrimination between asthmatics from healthy controls. Statistical analysis was performed using SPSS version 19.0.0. P <0.05 was considered significant.

RESULTS

Demographic and clinical parameters of the study subjects

We enrolled 140 healthy normal controls (NC) and 116 asthmatic patients, among which there were 50 that were assigned into the severe asthma (SA) group, while 66 were patients with non-severe asthma (NSA). There were no differences in age, BMI and sex distribution among the three groups. There was a higher rate of smokes among the asthmatic patients than in healthy controls (32.4 and 45.1 vs. 16.4%, both $p<0.05$). The SA group had longer asthma duration compared to the NSA group (9.56±4.38 vs. 14.37±8.56 years, $p=0.014$). The SA group had significantly lower baseline forced expiratory volume in 1 second (FEV1; 77.23±26.45 vs. 58.34±22.25, $p=0.012$) compared to the NSA group. The asthmatics had dramatically elevated serum total IgE level and serum hs-CRP levels than normal controls. In addition, the SA patients had even more increased total IgE level, serum hs-CRP levels than NSA patients (Table 1).

Association between APE1 and asthma

Compared to the NC group, the serum APE1 level in the NSA and SA group were higher than that in control group. However, no significant difference was noted between the NSA and SA groups, as shown in Fig. 1A. Similarly, we found that APE1 mRNA in peripheral blood eosinophils of NSA and SA patients were significantly increased in comparison to control groups. The SA group had slightly higher eosinophil APE1 mRNA level in contrast to NSA patients, but did not reach statistical significance (Fig. 1B). As for APE1 mRNA in peripheral blood neutrophils, we observed it was significantly upregulated in NSA and SA groups compared to controls. Noticeably, the SA patients also had a dramatically higher level of APE1 than NSA patients (Fig. 1C).

We also performed the Pearson's correlation analysis and found that APE1 mRNA levels of neutrophils of peripheral blood were significantly correlated with the other clinical indices, such as hs-CRP and Fev1%, as shown in Table 2. The serum APE1 and mRNA level in eosinophils are not correlated to the levels of hs-CRP and FEV1% pred. None of the three was correlated to total Ig E level.

Table 1
Demographic and clinical parameters of the study subjects.

Index	Controls (n=140)	NSA (n=50)	SA (n=66)
Age	42.43±7.23	41.45±10.31	46.63±11.45
Male (%)	51	54	48
BMI(kg/m^2)	24.37±3.14	25.52±3.67	25.72±4.43
Smoking rate(%)	16.4	32.4*	45.1*#
asthma duration (years)	-	9.56±4.38	14.37±8.56 #
Allergic history (%)	13	25*	27*#
Blood eosinophils(×10^9/L)	0.18±0.13	0.25±0.11	0.35±0.14#
Blood neutrophils (×10^9/L)	4.11±1.04	4.44±1.23	5.67±3.14#
Serum Hs-CRP	0.45±0.01	11.12±6.67*	14.12±6.45 *#
FEV1% pred	-	77.23±26.45	58.34±22.25*
Serum total IgE(IU/mL)	68.16±23.52	199.17±45.87	621.45±133.59*

BMI, Body mass index; Serum Hs-CRP, high-sensitivity C-reactive protein; FEV1% pred, forced expiratory volume in one second % of predicted value. * vs control, p<0.05; #, vs NSA, p<0.05; SA, severe asthma; NSA, Non-severe asthma.

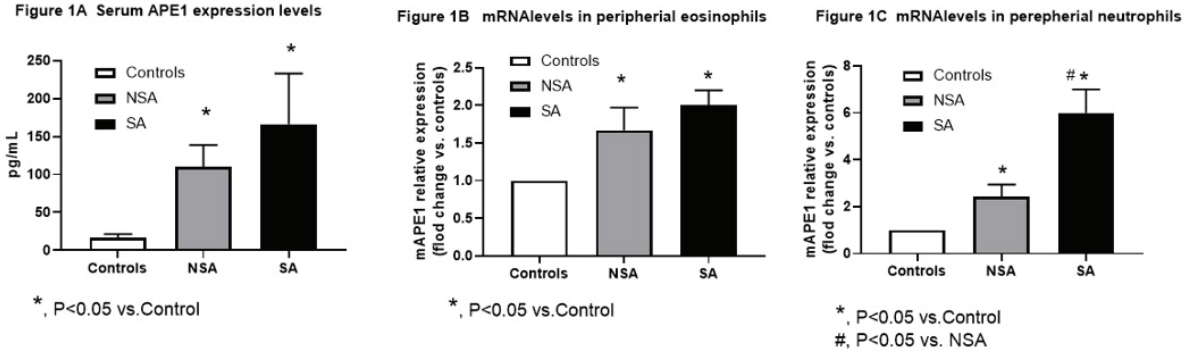

Fig. 1A. Serum APE1 levels detected using ELISA in control, non-severe asthma (NSA) and severe asthma (SA) groups by using the ANOVA test. **Fig. 1B.** APE1 mRNA in peripheral blood eosinophils using Realtime PCR assays in control, NSA and SA groups. **Fig. 1C.** APE1 mRNA in peripheral blood neutrophils using Realtime PCR assays in control, NSA and SA groups.

Diagnostic value determined by ROC analysis

To test the diagnostic value of serum APE1 and its mRNA in eosinophils and neutrophils, we performed the Receiver Operating Characteristic (ROC) curve analysis. As shown in Fig. 2A, the peripheral blood neutrophil APE1 mRNA can distinguish asthmatic patients (NSA+SA) from healthy controls, at a cutoff value of 2.14, with the AUC of 0.893 (95% CI, 0.847-0.938; p<0.001, with 87.5% sensitivity and 84.6% specificity). We next tested if neutrophil APE1 mRNA is related to the asthma severity. As shown in Fig. 2B, the APE1 mRNA at a cutoff value of 4.24, is adequate to identify SA subject from NSA subjects, with an AUC of 0.759 (95% CI, 0.674-0.846; p<0.001, 83.4% sensitivity and 80.3% specificity). On the other hand, the serum APE1 and eosinophil mRNA level,

Table 2
The correlation between APE1 protein or mRNA levels with the hs-CRP, Total Ig E and FEV1%.

	hs-CRP	Total Ig E	FEV1% pred
Serum APE1	R2=0.562, P=0.032	R2=0.223, P=0.115	R2=-0.307, P=0.055
Eosinophils APE1 mRNA	R2=0.215, P=0.084	R2=0.3632, P=0.064	R2=-0.103, P=0.774
Neutrophils APE1 mRNA	R2=0.775, P=0.003	R2=0.326, P=0.076	R2=-0.708, P=0.001

Serum Hs-CRP, high-sensitivity C-reactive protein; FEV1% pred, forced expiratory volume in one second % of predicted value.

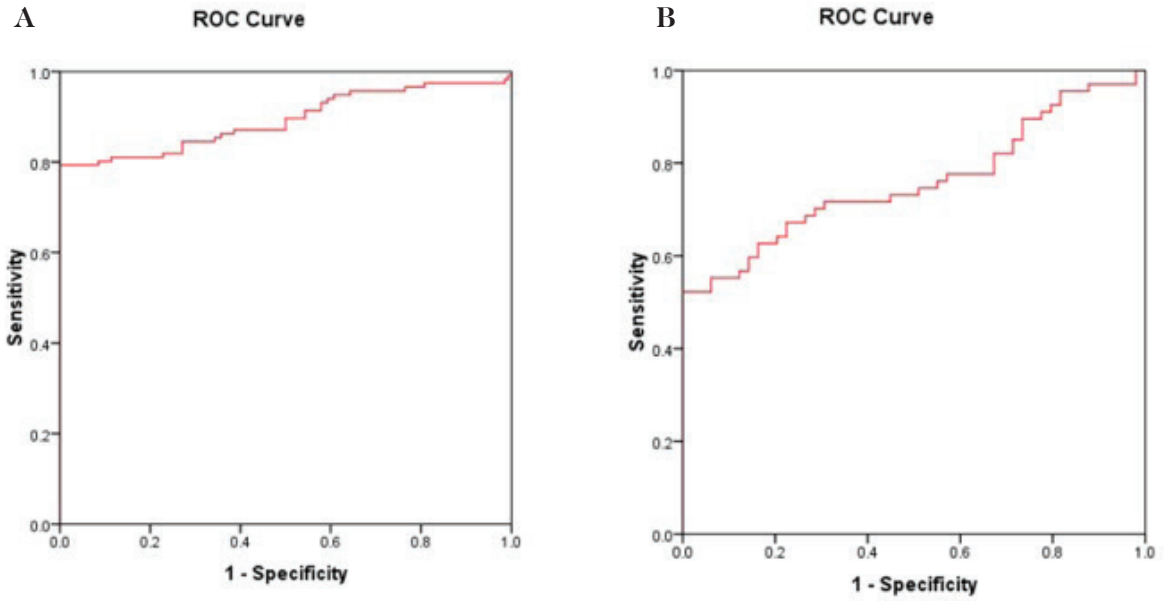

Fig. 2A. The Receiver-Operating Characteristic (ROC) analysis of APE1 mRNA in eosinophils of peripheral blood in distinguishing asthmatic patients (NSA+SA) from healthy controls, with an area under the curve value of 0.893 (95% Confidence Interval, 0.847-0.938; $p < 0.001$, with 87.5% sensitivity and 84.6%). **Fig. 2B.** The Receiver-Operating Characteristic (ROC) analyses of APE1 mRNA in eosinophils of peripheral blood distinguishing asthmatic patients NSA from SA groups, with an area under the curve value of 0.759 (95% Confidence Interval, 0.674-0.846; $p < 0.001$, 83.4% sensitivity and 80.3% specificity.

however, did not show a diagnostic difference in separating asthmatic patients from controls, nor are they related to the asthma severity (data not shown).

DISCUSSION

In this study, we detected the serum APE1, the peripheral blood eosinophil and neutrophil APE1 mRNA in adult asthmatic patients and healthy controls. We found although all of these markers were increased in asthmatic patients, only neutrophil APE1 mRNA has diagnostic significance in distinguishing asthmatics from controls, and also in separating severe patients from non-severe patients. This finding establishes, for the first time the association between APE1 and asthma, and also provides an easily accessible biomarker to evaluate the asthma development and severity in a clinical setting. To the best of our knowledge, we are

the first to confirm the association between APE1 and asthma.

APE1 has been increasingly viewed as a potent inflammatory regulator in a variety of inflammatory processes. In psoriatic skin, APE1 was markedly up-regulated in epidermal layers. APE1 the transcriptionally activated hypoxia-inducible factor-1α and NF-κB, two crucial transcription factors responsible for inflammation in keratinocytes. APE1 is essential for the expression of inflammatory cytokines and chemokines in HaCaT cells and primary keratinocytes [16, 17]. In ApoE-/- mouse model of atherosclerosis, plasma APE1 correlates with Atherosclerotic Inflammation levels and APE1/Ref-1 expression was upregulated in aortic tissues [18]. In macrophages, pharmacological inhibition of APE1 with its redox function inhibitor suppresses inflammatory response in activated macrophages [19]. Elevation of Serum APE1 was reported in experimental murine model for myocarditis [11].

APE1 has been used as a prediction marker of Environmental Carcinogenesis Risk, including smoking [20]. Smoking can in induce a various types of DNA damage and prompts cancers. Several previous studies reported the association between genetic variability of APE1 with lung cancer. Some researchers reported that APE1 genotypes were correlated with the risk of lung cancer among smokers [21], while the others reported that APE1 polymorphisms of −656T > G located in the promoter region and D148E are closely associated with lung cancer risk under cigarette smoking exposure [22, 23]. Smoking has been shown to exacerbate asthma severity by aggravating inflammation [24, 25]. Consistent with this, in our study, we observed that the severe asthma patients have a higher smoking status than non-severe asthma and healthy controls. The smoking amount is positively associated with the asthma severity (data not shown). However, the role of APE1 in asthma has not been elucidated so far.

Our study, for the first time, confirms the diagnostic significance of APE1 mRNA in peripheral blood neutrophils. Airway inflammation in bronchial asthma is characterized by infiltration with eosinophils and neutrophils [26, 27]. That was the reason we detected APE1 mRNA levels from eosinophils and neutrophils. Compared to the sputum, human peripheral blood is a stable source of eosinophils and neutrophils. Our data suggests APE1 mRNA in peripheral blood neutrophils, rather than that in eosinophils, can be used as a biomarker. Since we enrolled adult asthmatics, we cannot exclude the role of eosinophils APE1 m RNA in asthmatic children. To our surprise, our data did not reveal the clinical significance of serum APE1 for asthma diagnosis and classify its severity. In conclusion, our study discovered an easily accessible biomarker for asthma evaluation. Of course, further validation of our finding with a larger scale of sample size is needed.

ACKNOWLEDGEMENTS

This study was supported by a grant from Shanghai Science and Technology Commission (21142203600).

Conflict of interest

None.

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Contributions of authors

QJ conceived the study. ZH and QJ enrolled patients, collected patient's information and performed the lab assays. QJ analyzed the data and drafted manuscript.

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Invest Clin 63(4): 353 - 362, 2022 https://doi.org/10.54817/IC.v63n4a03

The effects of curcumin on the biological behavior of colorectal cancer cells through the JAK/STAT3 and RAS/MAPK/NF-κB pathways.

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Keywords: human colorectal cancer cells; HCT116 cells; growth cycle; proliferation; apoptosis.

Abstract. The purpose of this work was to investigate the effects of curcumin on the biological behavior of colorectal cancer cells through the JAK/STAT3 and RAS/MAPK/NF-κB pathways. Human colorectal cancer HCT116 cells were cultured and divided into a control group and low, medium and high-dose curcumin groups (n =5). HCT116 colorectal cancer cells became long-growing cells after incubation and culture at 37°C. The control group was treated with 15μL phosphate-buffered saline, and the low-dose, medium-dose and high-dose curcumin groups were treated with 20, 40 and 80μmol/L curcumin, respectively. All groups were treated with relevant drug intervention, digested and centrifuged for 48h, washed twice with a PBS solution, centrifuged at 1000 rpm for 3 min, and the cells precipitated. The proliferation, apoptosis and growth cycle of cells in each group were observed, and the expressions of the JAK/STAT3 and RAS/MAPK/NF-κB pathways and related proteins in each group were studied. Compared with the curcumin low-dose and medium-dose groups, the proliferation ability of the curcumin high-dose group was significantly decreased ($P<0.05$). When the low-dose and medium-dose curcumin groups were compared with the high-dose curcumin group, the apoptosis ability was significantly increased ($P<0.05$). When the low-dose and medium-dose curcumin groups were compared, the growth ratio of the G0/G1 phase in the high-dose curcumin group was significantly increased, and the percentage of the S phase was significantly decreased ($P<0.05$). Compared with the curcumin low-dose and medium-dose groups, the expression of JAK-STAT3 and RAS/MAPK/NF-κB pathway in the curcumin high-dose group was significantly decreased ($P<0.05$). The protein expressions of STAT3, RAS, P-P38 and P65 in the curcumin high-dose group were significantly lower than those in the curcumin low-dose and medium-dose groups ($P<0.05$). Curcumin can inhibit the expression of JAK/STAT3 and RAS/MAPK/NF-κB pathways, block the growth cycle, and inhibit the proliferation and induce apoptosis of colorectal cancer cells, providing a new idea for the clinical treatment of colorectal cancer.

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Los efectos de la curcumina en el comportamiento biológico de las células del cáncer colorrectal mediante las vías JAK/STAT3 y RAS/MAPK/NF-KB.

Invest Clin 2022; 63 (4): 353 – 362

Palabras clave: células de cáncer colorrectal humano; células HCT116; ciclo de crecimiento; proliferación; apoptosis.

Resumen. El objetivo del presente trabajo fue investigar los efectos de la curcumina en el comportamiento biológico de las células del cáncer colorrectal mediante el estudio de las vías JAK/STAT3 y RAS/MAPK/NF-KB. Las células del cáncer colorrectal humano HCT116 se cultivaron y dividieron en un grupo control y en grupos con dosis baja, media y alta (n = 5) de curcumina. Las células de cáncer colorrectal HCT116 se convirtieron en células de crecimiento prolongado después de la incubación y cultivo a 37°C. El grupo de control se trató con 15 μL de solución tampón fosfato salina (PBS) y los grupos de curcumina de dosis baja, media y alta se trataron con 20, 40 y 80 μmol/L de curcumina, respectivamente. Todos los grupos fueron tratados con la intervención farmacológica pertinente, digeridos y centrifugados durante 48 horas, lavados dos veces con solución de PBS, centrifugados a 1000 rpm durante 3 minutos, y las células precipitadas. Se observaron la proliferación, la apoptosis y el ciclo de crecimiento de las células de cada grupo, y fueron estudiados las expresiones de las vías JAK/STAT3, RAS/MAPK/NF-KB y proteínas relacionadas en cada grupo. Comparado con los grupos de la dosis baja y media de la curcumina, disminuyó obviamente la capacidad de proliferación del grupo de la dosis alta de la curcumina (P<0,05). Comparado con los grupos de la dosis baja y media de la curcumina, aumentó de modo significativo la capacidad de la apoptosis del grupo de la dosis alta de la curcumina (P<0,05). Comparado con los grupos de la curcumina de dosis baja y media, aumentó obviamente la proporción del crecimiento de la fase G0/G1 en el grupo de la curcumina de dosis alta y el porcentaje de la fase S disminuyó considerablemente (P<0,05). Las expresiones proteicas STAT3, RAS, P-P38 y P65 en el grupo de la dosis alta de la curcumina fueron evidentemente más bajas que las de los grupos de la dosis baja y media de la curcumina (P<0.05). La curcumina puede inhibir la expresión de las vías JAK/STAT3 y RAS/MAPK/NF-KB, bloquear el ciclo del crecimiento y luego inhibir la proliferación e inducir apoptosis de las células del cáncer colorrectal, lo que brinda una nueva idea para el tratamiento clínico del cáncer colorrectal.

Received: 30-04-2022 Accepted: 04-06-2022

INTRODUCTION

Colorectal cancer is a common gastrointestinal malignancy in clinical practice, ranking third among malignancies worldwide, and its mortality rate is second only to liver cancer and lung cancer [1]. Data survey shows that the incidence and mortality of colorectal cancer are on the rise, and its mortality rate ranks second among malignant tumors in developed countries, seriously threatening human health and quality of life [2]. Studies have

found that the incidence of colorectal cancer is gradually rising in younger people, and the prognosis of patients underyears old-old is poor [3]. Surgical resection is currently the primary method treatingt for colorectal cancer in clinical practice. Patients in advanced stages are mostly treated with radiotherapy and chemotherapy, but the treatment effect of colorectal cancer is not ideal; and the prognosis of the patients is poor, and most patients suffer from recurrence and metastasis [4]. Curcumin is a natural and effective chemical component in plants. It mainly exists in the roots and stems of turm,eric and it is insoluble in water. It can change with the change of acid and alkali conditions. Curcumin is widely used in food production as a preservative, colorant, etc., and also plan essentialtant role in anti-tumor, hypolipidemic, anti-oxidation and anti-arteriosclerosis[5]. Jabus kinases/signal and activator of the transcription Jak-stat pathway is involved in the cycle, cell transformation and apoptosis of tumor cells [6]. It was found [7] that Rat sarcoma (RAS) protein can activate the mitogen activated protein kinase (MAPK) and the nuclear factor (NF-κB) pathway. It can induce the proliferation of hepatocellular carcinoma cells and strengthen their invasion ability. However, curcumin has been rarely studied in the JAK/STAT3 and RAS/MAPK/NF-κB pathways of colorectal cancer. Therefore, this study aimed to explore the effects of curcumin on the biological behavior of colorectal cancer cells through the JAK/STAT3 and RAS/MAPK/NF-κB pathways.

MATERIAL AND METHODS

Experimental materials

Human colorectal cancer HCT116 cells were purchased from Hubei Punosai Life Science and Technology Co., LTD., and were uniformly cryopreserved by the experimental center of our hospital.

Experimental instruments and reagents

Curcumin (Beijing Green Heng Xing Biotechnology Co., LTD.), PBS buffer (Beijing Standard Technology Effective Company), medium (Shanghai Biotechnology Co., LTD.), fetal calf serum (Jiangsu Ke Wei Biotechnology Co., LTD.), dimethyl sulfoxide (Chengdu Medical Technology Co., LTD.), automatic labeling instrument (Shanghai Molecular Instrument Co., LTD.,), protein extraction kit (Beijing Solaibao Technology Co., LTD.), flow cytometer (Shanghai Huanlian Medical Device Co., Ltd.).

Cell Grouping

Colorectal cancer HCT116 cells were placed in 10% fetal bovine serum and cultured in a cell incubator with 5% CO_2 and 37°C. When the cells were fused to 90%, a trypsin solution was given for digestion and passage treatment. When the passage reached the third generation, cells with long-term growth were selected for experiment. In the process of culture, the growth state of cells was observed, and the conventional fluid was changed according to its state. Colorectal cancer HCT116 cells were divided into control group, and a curcumin low-dose, medium-dose and high-dose groups. The control group was treated with 15μL phosphate buffered saline, and the low-dose, medium-dose and high-dose curcumin groups were treated with 20, 40 and 80 μmol/L curcumin, respectively, to observe the proliferation and apoptosis of cells in each group. The expression of JAK/STAT3, RAS/MAPK/NF-κB pathway and related proteins were studied in each group.

EXPERIMENTAL METHODS

Cell proliferation experiment

The cell cycle is the most important entity for cell survival. Abundant factors and proteins in positive or negative maps at multiple points and bottlenecks, precisely and harmoniously regulate and control this cycle. In fact, there are a variety of genes in cells that encode proteins needed to control the cell cycle. Although the cell cycle is monitored and inspected at several stations,

this adjustment takes place especially at two points with extraordinary intensity and care. The cell, first, decides to replicate its own DNA, and second, to initiate mitotic division. These steps are in the realm of passing from G1 to S and from G1 to M.

The cells in each group were successively inoculated into a 96-well culture plate, and the cell density was adjusted to 10×10^4/mL. The cells were observed for 5h, and the corresponding intervention was given to each group. Cultured again for 36 h, the MTT assay was used to observe the situation of each group of cells, and the proliferation of each group was plotted. The experiment was repeated 3 times.

Flow cytometry detection

Colorectal cancer HCT116 cells with a concentration of 2×10^8/L were inoculated into 6-well culture plates for 12 h. The control group was given routine culture, and the curcumin low-dose, medium-dose and high-dose groups were given 20, 40 and 80 μmol/L curcumin for intervention. After 48 h of intervention, the cells were collected and centrifuged at 1000 rpm for 10 min. The cells were washed with PBS twice, and 1ml of precooled 70% ethanol was used for beating and dispersing. The cells were fixed overnight at -20°C, and then mixed with 0.8 μg/mL. The cells were incubated at room temperature for 30 min without light.

Western blot assay

Western blot analysis was performed essentially according to standard protocol. Briefly, the cells were solubilized in lysis buffer (50 mM Tris, 100 mM NaCl, 2.5 mM EDTA, 1% Triton X-100, 1% Nonidet P-40, 2.5 mM Na3O4V, 25 μg/mL aprotinin, 25 μg/mL leupeptin, 25 μg/mL pepstatin A, and 1 mM phenylmethylsulfonyl fluoride). After clarification at 10,000g for 15 minutes, the supernatant was used for Western blot analysis. In all analyses, protein concentration, determined by the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA), was standardized among the samples. Aliquots of cell lysates containing 50 μg of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred electrophoretically onto supported nitrocellulose membranes (Osmonics, Gloucester, MA). Membranes were incubated for 1 hour at room temperature with blocking buffer, TBS-T (20 mM Tris, pH7.6, 100 μMNaCl, 0.1% Tween-20) and 5% nonfat dry milk with gentle agitation. After washing the membranes with TBS-T, they were incubated overnight at 4°C in TBS-T buffer containing antibody dilution buffer as suggested by the manufacturer and with antibodies (1:1000 dilution) to CD44, CD166 (Santa Cruz Biotechnology, Santa Cruz, CA), or epidermal growth factor receptor (EGFR; Cell Signaling, Beverly, MA). The membranes were washed three times with TBS-T and subsequently incubated with appropriate secondary antibodies (1:5000 dilutions) in TBS-T containing 5% milk for 1 to 2 hours at room temperature with gentle agitation. The membranes were washed again with TBS-T, and the protein bands were visualized by enhanced chemiluminescence (ECL) detection system (Amersham, Piscataway, NJ). The membranes containing the electrophoresed proteins were exposed to X-Omat film (Sigma-Aldrich, St Louis, MO). The membranes were stripped (twice × for 15 minutes at 55°C) in stripping buffer containing 100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, and 62.5 mM Tris-HCl pH 6.7, and reprobed for β-actin using the corresponding antibodies, which were used as a loading control. All Western blots were performed at least three times for each experiment [8]. The method used to identify proteins in the membrane is the blotting method. In this technique, protein bands are transferred from the gel to a nitrocellulose membrane that can bind and stabilize proteins. To do this, by blotting, the protein molecules were removed from the gel and placed in the same position on the surface of the membrane, so we could easily study them, separate

them, and finally use them. Specific ligands or substrates were used to detect proteins or enzymes transferred to the membrane. Antibodies were also used to specifically detect proteins in the membrane.

All groups were treated with relevant drug intervention, digested and centrifuged for 48h, washed twice with PBS solution, centrifuged at 1000 rpm for 3min, and the cells precipitated. The protein was extracted, 200μL lysate was put into the cell sample, let stand and mix. The BCA method was used to determine protein content according to the kit instructions. After gel preparation, sample loading, electrophoresis, membrane transfer, elution, sealing, primary antibody incubation, membrane washing, secondary antibody incubation and membrane washing again, the protein expression was calculated by the Invitrogen™ /Thermo Fischer Scientific, USA, technique.

Apoptosis methodology

There are two pathways for cell apoptosis, intrinsic and external. For this study, the TUNEL method was used to evaluate apoptosis [9].

To diagnose apoptosis, the TUNEL kit (insitu cell death detection kit, POD, Roche company, made in Germany) (Cat. No. 11 684 817 910) was used. The TUNEL technique was performed as follows:

1. First, the prepared sections were washed with para-proteinase K after paraffinization and irrigation, and after incubation, they were washed with buffer phosphate solution for 30 minutes at 37°C.

2. Tissue sections were stained with 50 μl of TUNEL reaction solution for 37 minutes at 37°C, then washed with buffer phosphate solution.

3. At this stage, the sections were then washed with a converter solution (50 μL) for 30 minutes at 37°C with buffer phosphate solution and then mixed with a diamino benzidine solution for 20 minutes and then stirred for 25 minutes. They were incubated again at 37°C.

4. The sections were then washed three times with phosphate solution and batoloidin blue, for two minutes each time.

Cells were manually examined in 10 random high-power (×100 magnification) fields (>1000 cells) and the apoptosis index (AI) was determined as follows: AI = number of positively stained cells/total number of cells counted.

Statistical methods

The SPSS20.0 software package was used for statistical analysis of the study data, and the measurement data was expressed as means ± standard deviation ($\bar{x} \pm s$). One way-ANOVA was used for comparison between groups. $P<0.05$ was taken as the statistical standard.

RESULTS

Comparison of proliferation ability of different groups of cells

Compared with the control group, the proliferation ability of the medication group was significantly decreased, with statistical significance ($P<0.05$); compared with the low-dose and medium-dose curcumin groups, the proliferation ability of the high-dose curcumin group was significantly decreased, with statistical significance ($P<0.05$), as shown in Table 1.

Comparison of apoptosis in different groups

Compared with the control group, the apoptotic ability of the medication group was significantly increased ($P<0.05$); compared with the low-dose and medium-dose curcumin groups, the apoptotic ability of the high-dose curcumin group was significantly increased ($P<0.05$), as shown in Table 2.

Comparison of cell growth cycles of different groups

Compared with the control group, there was no significant difference in G2/M phase ($P>0.05$), but the growth ratio of G0/

Table 1
Comparison of migration ability (proliferation) of different groups of cells ($\bar{x} \pm s$)

Group	Number of stems	proliferation ability（%）
control group	5	96.44 ± 3.15
low-dose curcumin group	5	76.16 ± 5.33*
medium-dose curcumin group	5	59.13 ± 4.16*#
high-dose curcumin group	5	45.11 ± 3.85*#△
F		139.29
P		＜0.001

Compared with control group, *P<0.05; compared with curcumin low-dose group, #P<0.05; compared with curcumin medium-dose group, △P<0.05. P-Value based on One Way- ANOVA. Number of stems: number of groups of cells.

Table 2
Comparison of apoptosis in different groups ($\bar{x} \pm s$)

Group	Number of stems	Apoptosis (%)
control group	5	5.23 ± 1.32
low-dose curcumin group	5	14.35 ± 1.22*
medium-dose curcumin group	5	25.76 ± 1.68*#
high-dose curcumin group	5	35.79 ± 2.11*#△
F		337.77
P		<0.001

Compared with control group, *p < 0.05; compared with curcumin low-dose group, #p< 0.05; compared with curcumin medium-dose group, △p< 0.05. P-Value based on One Way- ANOVA. Number of stems: number of groups of cells.

G1 phase in the treatment group was significantly increased, and the percentage of S phase was significantly decreased, the difference was statistically significant (P<0.05). Compared with the low-dose and medium-dose curcumin groups, the growth ratio of G0/G1 phase in the high-dose curcumin group was significantly increased. The percentage of S stage decreased significantly, with statistically significant difference (P<0.05), as shown in Table 3.

Comparison of JAK-STAT3 and RAS/MAPK/NF-κB pathway expression in different groups

Compared with the control group, the expression of JAK-STAT3 and RAS/MAPK/NF-κB pathway in the treatment group was significantly decreased, and the differences were statistically significant (P<0.05).The expression of JAK-STAT3 and RAS/MAPK/NF-κB pathway (μg) was significantly decreased in the high-dose curcumin group, and the differences were statistically significant (P<0.05), as shown in Table 4.

Comparison of related protein expression in different groups

Compared with the control group, the protein expressions (μg) of STAT3, RAS, P-P38 and P65 in the medication group were significantly decreased, with statistical significance (P<0.05). Compared with the low-dose and medium-dose curcumin groups, the protein expressions of STAT3, RAS, P-p38 and P65 in the high-dose curcumin group were significantly decreased. The difference was statistically significant (P<0.05), as shown in Table 5.

Table 3
Comparison of cell growth cycles of different groups ($\bar{x} \pm s$)

Group	Number of stems	G0/G1(%)	S(%)	G2/M(%)
control group	5	33.26 ± 2.19	53.76 ± 3.11	11.52 ± 3.16
low-dose curcumin group	5	45.38 ± 2.56*	41.35 ± 2.94*	11.31 ± 2.25
medium-dose curcumin group	5	59.67 ± 3.12*#	30.85 ± 3.16*#	10.23 ± 1.35
high-dose curcumin group	5	71.31 ± 3.15*#△	20.33 ± 2.08*#△	9.22 ± 1.64
F		177.61	125.62	1.00
P		<0.001	<0.001	0.418

Compared with control group, *$p<0.05$; compared with curcumin low-dose group, #$p<0.05$; compared with curcumin medium-dose group, △$p<0.05$. P-Value based on One Way- ANOVA. Number of stems: number of groups of cells.

Table 4
Comparison of JAK-STAT3 and RAS/MAPK/NF-κB pathway expression (μg) in different groups ($\bar{x} \pm s$)

Group	Number of stems	JAK-STAT3	RAS	p38MAPK	NF-κB
control group	5	1.06±0.09	1.15±0.26	0.98±0.02	0.85±0.16
low-dose curcumin group	5	0.71±0.11*	0.73±0.09*	0.75±0.11*	0.54±0.05*
medium-dose curcumin group	5	0.51±0.06*#	0.53±0.05*#	0.42±0.07*#	0.40±0.05*#
high-dose curcumin group	5	0.31±0.02*#△	0.29±0.04*#△	0.26±0.03*#△	0.22±0.04*#△
F		32.07	33.24	114.71	43.99
P		<0.001	<0.001	<0.001	<0.001

Compared with control group, *$p<0.05$; compared with curcumin low-dose group, #$p<0.05$; Compared with curcumin medium-dose group, △$p<0.05$. P-Value based on One Way- ANOVA. Number of stems: number of groups of cells. Unit of protein values: μg.

Table 5
Comparison of related protein expression (μg) in different groups ($\bar{x} \pm s$)

Group	Number of stems	STAT3	ras	p-p38	p65
control group	5	1.31 ± 0.35	1.53 ± 0.41	1.35 ± 0.31	1.45 ± 0.15
low-dose curcumin group	5	0.92 ± 0.19*	1.02 ± 0.19*	0.85 ± 0.08*	0.73 ± 0.11*
medium-dose curcumin group	5	0.59 ± 0.06*#	0.62 ± 0.08*#	0.51 ± 0.06*#	0.52 ± 0.05*#
high-dose curcumin group	5	0.29 ± 0.02*#△	0.28 ± 0.01*#△	0.26 ± 0.01*#△	0.21 ± 0.03*#△
F		23.64	27.48	41.90	146.12
P		<0.001	<0.001	<0.001	<0.001

Compared with control group, *$p < 0.05$; compared with curcumin low-dose group, #$p< 0.05$; compared with curcumin medium-dose group, △$p< 0.05$. P-Value based on One Way- ANOVA. Number of stems: number of groups of cells. Unit of protein values: μg.

DISCUSSION

With the changes of diet structure and the improvement of living standards, the incidence of colorectal cancer has been high, and is closely related to genetics, the environment and diet. Data survey shows that the incidence of colorectal cancer in big cities in China is higher than that in other cities, and it shows an increasing trend year by year. Colorectal cancer has become the most common and fastest growing malignant tumor in China [10]. Surgery combined with radiotherapy and chemotherapy is commonly used in the clinical treatment of colorectal cancer, but about 50% of patients will have recurrence and metastasis after surgery, which will eventually lead to death of patients [11]. The application of chemotherapy drugs can lead to serious toxic and side effects in patients, waste medical resources, and bring serious economic burden to families and society. Therefore, finding effective and safe drugs to treat colorectal cancer has become the focus of clinical research.

Traditional Chinese medicine treatment has been gradually applied in clinical practice, and has unique advantages, and has become an important part of clinical tumor treatment. Studies have found [12] that curcumin widely exists in turmeric, which can be combined with chemoradiotherapy drugs to effectively reduce the toxic and side effects of chemotherapy drugs, the dosage of these drugs, and to improve the prognosis and the quality of life of patients. Curcumin has been applied to thyroid cancer cells, and inhibition of thyroid cancer cell proliferation was observed, which may be related to the inhibition of p-MTOR and P-S6K proteins in thyroid cancer [13]. Another study in thyroid cancer found that curcumin could down-regulate the expressions of cy-Clinbl and Bcl-XL, and then inhibit thyroid cancer cells, thereby promoting their apoptosis [14]. The results were similar to those of this group. The cell growth cycle is the most important step in tumorigenesis and development. Foreign studies have found that when curcumin is applied to colorectal cancer cells, it is found that G1 block occurs, and then the apoptosis specific DNA is delayed, suggesting that curcumin can regulate the growth cycle of colorectal cancer cells [15]. In this experiment, compared with curcumin low-dose and medium-dose groups, the proliferation ability of curcumin high-dose group was significantly decreased, and the apoptosis ability was significantly increased ($P<0.05$). These results suggest that curcumin can inhibit the proliferation of colorectal cancer cells, block the growth of colorectal cell cycle, and induce their apoptosis.

During the occurrence and development of gastric cancer, the Jak-Stat3 pathway and the RAS-MAPK pathway have been successively activated and have complex interactions, which are closely related to gastric cancer. MAPK is a core member of the RAS-MAPK pathway, which can transmit extracellular signals to the nucleus by activating MAPK, leading to activation of phosphoamino acid residues of Jun, FOS and other transcription factors in the nucleus, thus regulating gene expression and finally leading to cell growth and differentiation [16]. The terminal of STAT3-C has a serine residue, and Ser727 is the phosphorylation site of MAPK, indicating that STAT3 is closely related to p38MAPK. Studies have confirmed that STAT3 has a certain correlation with p38MAPK in gastric cancer, and the expression of STAT3 can affect the expression of p38MAPK, which increases with the increase of STAT3 [17]. RAS exists in the form of binding proteins in DNA and mutates in tumors, thus affecting the protein activity of RAS and accelerating the proliferation, migration and invasion of tumors, and has become an important target of cancer [18]. It was found that MAPK/NF-κB can act as a downstream pathway of RAS, leading to RAS activation, which in turn activates MAPK enzyme, resulting in transfer and phosphorylation of Raf molecules downstream of RAS, thereby activating MAPK, and activating NF-κB in the nucleus, amplifying

RAS activity. Ultimately, the inflammatory response is accelerated [19]. In this study, the expressions of JAK-STAT3 and RAS/MAPK/NF-κB pathways were significantly decreased in the curcumin high-dose group, and the protein expressions of STAT3, RAS, P-P38 and P65 were significantly decreased in curcumin low-dose and medium-dose groups ($P < 0.05$). These results suggest that curcumin can inhibit the expression of JAK-STAT3 and RAS/MAPK/NF-κB pathways, further inhibit related proteins, and prevent further proliferation of colorectal cancer cells.

In this experimental study, curcumin could inhibit the proliferation and induce apoptosis of colorectal cancer cells by inhibiting the expression of JAK/STAT3 and RAS/MAPK/NF-κB pathways and block the growth cycle, providing a new idea for clinical treatment of colorectal cancer.

Conflict of interest

The manuscript has no conflict of interest.

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Limitation

Low number of groups of cells.

Authors Contribution

ZY and RZhao collected the samples. ZY and WG analysed the data. RZ and WG conducted the experiments and analyzed the results. All authors discussed the results and wrote the manuscript.

Funding

None.

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Invest Clin 63(4): 363 - 375, 2022 https://doi.org/10.54817/IC.v63n4a04

Relationship between peripheral arterial disease severity determined by the Glass classification and triglyceride-glucose index; novel association and novel classification system.

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Keywords: peripheral arterial disease; GLASS classification; triglyceride-glucose index; insulin resistance.

Abstract. Peripheral arterial disease is a serious clinical manifestation caused by atherosclerosis. It is one common cause of morbidity and mortality worldwide. It is commonly seen in males, and its (prevelance) increases with age. It is most prevalent with smoking, hypertension, diabetes mellitus and hyperlipidemia. Novel studies investigate the relationship between triglyceride-glucose index (TyG) and cardiovascular diseases. Studies investigating the association of this index and peripheral arterial disease and disease severity are generally done by using The Trans-Atlantic Inter-Society Consensus (TASC) classification. We aimed to study this association by using the new Global Limb Anatomic Staging System (GLASS) classification. Two hundred patients between 25 to 90 years old diagnosed with peripheral arterial disease and admitted to the hospital for peripheral arterial angiography between July 2021 and December 2021, were evaluated retrospectively with blood parameters and angiographic images. Patients were divided into two groups: moderate (group 1; n=58) and severe (group 2; n=142) according to the GLASS classification. No statistical differences were observed for comorbidities and repeated interventional procedure rates (p=0.164). Triglyceride values were found to be statistically different between groups (p=0.040). TyG was found higher in group 2 (p= 0.04). According to the binary logistic regression model, only TyG was found to have a significant effect as a diagnostic factor (p=0.011). TyG was also significantly correlated with the Rutherford (p=0.012) and GLASS classification severity (p<0.001). Peripheral arterial disease and disease severity could be easily monitored with simple calculable TyG. In this way, precautions could be taken, and morbidities could be prevented.

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Relación entre la gravedad de la enfermedad arterial periférica determinada por la clasificación GLASS y el índice de triglicéridos-glucosa; nueva asociación y nuevo sistema de clasificación.

Invest Clin 2022; 63 (4): 363 – 375

Palabras clave: enfermedad arterial periférica; clasificación de GLASS; indice de triglicéridos-glucosa; resistencia a la insulina.

Resumen. La enfermedad arterial periférica es una manifestación clínica importante causada por la aterosclerosis. Es una causa común de morbilidad y mortalidad en todo el mundo. Se ve comúnmente en hombres y la prevalencia aumenta con la edad. Es más común con el tabaquismo, la hipertensión, la diabetes mellitus y la hiperlipidemia. Nuevos estudios investigan la relación entre el índice de triglicéridos-glucosa (TyG) y las enfermedades cardiovasculares. Los estudios que investigan la asociación de este índice y la enfermedad arterial periférica generalmente se realizan utilizando la clasificación de TASC. Nuestro objetivo fue estudiar esta asociación utilizando la nueva clasificación de GLASS (sistema global de estadificación anatómica de extremidades). Doscientos pacientes entre 25 a 90 años con diagnóstico de enfermedad arterial periférica e ingresados al hospital para angiografía arterial periférica entre julio de 2021 y diciembre de 2021, fueron evaluados retrospectivamente con parámetros sanguíneos e imágenes angiográficas. Los pacientes se dividieron en dos grupos: leves (grupo 1; n=58) y graves (grupo 2; n=142) según la clasificación de GLASS. No se observaron diferencias estadísticas para las comorbilidades y las tasas de procedimientos intervencionistas repetidos (p = 0,164). Los valores de triglicéridos se encontraron significativamente diferentes entre los grupos (p= 0,04). El índice de triglicéridos-glucosa se encontró más alto en el grupo 2 (p= 0,04). Según el modelo de regresión logística binaria, solo el índice de triglicéridos-glucosa resultó tener un efecto significativo como factor diagnóstico (p=0,011). El índice de triglicéridos-glucosa también se correlacionó significativamente con la gravedad de la clasificación de Rutherford (p=0,012) y la clasificación de GLASS (p<0,001). La enfermedad arterial periférica y la gravedad de la enfermedad podrían controlarse fácilmente con TyG calculable simple. De esta manera, se podrían tomar precauciones y prevenir morbilidades.

Received: 14-03-2022 Accepted: 20-07-2022

INTRODUCTİON

Peripheral arterial disease (PAD) is an important disease that arises from systemic atherosclerosis and has effected about 200 million people worldwide [1,2]. Although PAD is most commonly seen among males, its incidence in women has increased especially at ages over 50 years [3]. Its prevelance incereases with age, and the reported rate is about 20% or above in individuals who are over 80 years [4]. It is a pathology that could result in

morbidities ranging from lower extremity ulcers to limb losses. Despite the high morbidity rates, PAD tends to be asymptomatic until it reaches the advanced stage [5]. Its presentation can vary from asymptomatic phase to critical limb ischemia (CLI) and most of the patients that are admitted to hospitals suffer from intermittent claudication. The most common diagnostic test for PAD is ankle-brachial index (ABI) measurement and values ≤ 0.90 is considered as arterial stenosis and its sensitivity in diagnosing PAD is 95% [6,7]. Doppler ultrasonography generally is the first imaging method choosed for diagnosing PAD.

Despite this type of clinical course and well-known adverse outcomes, the pathophysiology of PAD has not yet been fully understood. The well-known main underlying pathology of PAD is atherosclerosis [8]. Since atherosclerosis is a common underlying pathology, the risk factors for PAD are; hypertension (HT), smoking, diabetes mellitus (DM) and hyperlipidemia [9].

Insulin resistance (IR), is usually one of the main events underlying DM, a pathology characterized by decreased insulin sensitivity of peripheral tissues [10] and the resulting chronic hyperinsulinemia is significantly associated with atherosclerotic cardiovascular disease (CVD) [11-13]. It has been detected that insulin-resistant patients endure higher cardiovascular risk than insulin-sensitive subjects [14]. According to these reports, an association with IR and vascular disease is very likely but exact pathogenesis of this relation remain controversial. The effect on the vascular area is one of the pathological mechanism evaluated in the association between DM and PAD [15]. IR related vascular damage includes functional and structural vascular injury such as; vascular wall elasticity loss (arterial stiffness), increased intima-media thickness, impaired vasodilation and vascular calcification [16].

Also the relationship between the triglycerides (TGs), CVD and atherosclerosis is still controversial. Recent studies have provided evidence that TGs and TG-rich lipoproteins are among the causes of CVD [17].

The triglyceride-glucose index (TyG), a calculated index by using fasting blood glucose and triglyceride values, has been defined as a novel marker of IR [18-20] and studies have shown a relationship between the TyG and CVD, stroke, carotid atherosclerosis and coronary artery disease (CAD) [21-23]. Although many studies have evaluated the association of this index with CAD, CVD and carotid atherosclerosis, there are currently very few data about its association with PAD and disease severity. We aimed to investigate the association between TyG and PAD severity, by using a new anatomical classification for PAD named Global Limb Anatomic Staging System (GLASS).

MATERİAL AND PATİENTS

Our study is a retrospective observational comparative study that compares results of individuals allocated into two groups according to the severity of their lesions. Two hundred patients that were between 25 and 90 years old, admitted to our outpatient clinic, diagnosed with PAD and hospitalized for peripheral arterial angiography between July 2021-December 2021 were investigated retrospectiveley. Patient data were obtained from Isparta City Hospital's hospital registration system and angiography laboratory archive. The study was approved by Suleyman Demirel University Medical Faculty Ethical Committee (Number:72867572-050.01.04-196235).

Patients that were admitted to outpatient clinic with intermittant claudication or extremity ulcers, diagnosed with PAD as a result of clinical examination (absence of palpable peripheral pulses) and low ABI measurement (ABI≤ 0.9). The Rutherford Classification was used for clinical staging of existing disease. According to Rutherford Classification, asymptomatic patients staged as "Cathegory 0", mild claudication as "Cathegory 1", moderate claudication as "Cathegory 2", severe claudication staged

as "Cathegory 3", ischemic rest pain as "Cathegory 4", minor tissue loss "Cathegory 5" and major tissue loss expressed as "Cathegory 6" [24].

Patients diagnosed with PAD as a result of clinical examination were taken to color Doppler ultrasonography (Toshiba Applio 500; Japan) and if a pathology was detected, Computed Tomographic Angiography (CTA) (Hitachi Supria; Japan) was applied for detailed evaluation. After performing CTA, patients that had peripheral arterial stenosis more than 50% were hospitalized for peripheral arterial angiography for further evaluation and treatment. Angiographies performed in Isparta City Hospital angiography laboratory (Toshiba Infinix; Japan) by the same physician with local anesthesia from right or left femoral artery approaches with 6F sheath by using Seldinger technique. Antegrad or retrograd approaches selected according to the lesions of the patients evaluated by CTA images.

Patients who underwent peripheral angiography were evaluated by novel GLASS anatomic classification in terms of PAD severity. (Table 1) GLASS classification is a new anatomic classification system using angiographic findings for severity of PAD. It was published in 2019 by joining of three

Table 1
Global Limb Anatomic Staging System (GLASS).

Aorta-Iliac Grading	
1	Stenosis of the common and external iliac artery, chronic total occlusion of either common or external iliac artery (not both), stenosis of the infrarenal aorta; any combination of these.
2	Chronic total occlusion of the aorta; chronic total occlusion of common and external iliac arteries; severe diffuse disease and/or small-caliber (<6 mm) common and external iliac arteries; concomitant aneurysm disease; severe diffuse in-stent restenosis in the aorta-iliac system.
Femoro-Popliteal (FP) Grading	
0	Mild or no significant (<50%) disease
1	Total length SFA disease <1/3 (<10 cm); may include single focal CTO (<5 cm) as long as not flush occlusion; popliteal artery with mild or no significant disease.
2	Total length SFA disease 1/3–2/3 (10–20 cm); may include CTO totaling <1/3 (10 cm) but not flush occlusion; focal popliteal artery stenosis <2 cm, not involving trifurcation.
3	Total length SFA disease >2/3 (>20 cm) length; may include any flush occlusion <20 cm or non-flush CTO 10–20 cm long; short popliteal stenosis 2–5 cm, not involving trifurcation.
4	Total length SFA occlusion >20 cm; popliteal disease >5 cm or extending into trifurcation; any popliteal CTO.
Infra-Popliteal (IP) Grading	
0	Mild or no significant (<50%) disease.
1	Focal stenosis <3 cm not including TP trunk.
2	Total length of target artery disease <1/3 (<10 cm); single focal CTO (<3 cm not including TP trunk or target artery origin).
3	Total length of target artery disease 1/3–2/3 (10–20 cm); CTO 3–10 cm (may include target artery origin, but not TP trunk).
4	Total length of target artery disease >2/3 length; CTO >1/3 (>10 cm) of length (may include target artery origin); any CTO of TP trunk.

Table 1
CONTINUATION

Inframalleolar/Pedal Grading	
0	Target artery crosses ankle into foot, with intact pedal arch
1	Target artery crosses ankle into foot; absent or severely diseased pedal arch
2	No target artery crossing ankle into foot

CTO: Chronic Total Occlusion; SFA: Superficial Femoral Artery; TP:Tibio-peroneal.

vascular societies [25]. With this new anatomic staging system, better assessment of limb ischemia and better characterization of the anatomic specifications of vascular disease could be achieved [26].

After 12 hours of overnight fasting, venous blood samples for biochemical and hematological parameter measurements were taken from the blood drawn from the antecubital vein at the first day after hospital admission for blood analysis. Biochemical analysis included the serum lipid profile and fasting glucose levels (Variant 2-Turbo, Bio-Rad; Japan).

TyG is calculated as Ln (fasting triglycerides (mg/dL) × fasting glucose (mg/dL) / 2). ABI was calculated as the ratio of highest systolic ankle pressure to highest systolic brachial pressure measured manually.

Patients diagnosed with Buerger disease, vasculitis, acute limb ischemia, systemic inflammatory diseases, chronic liver and hematological diseases; patients operated or had vascular interventions before outpatient clinic admission; patients who have known malignancy and whose index could not be calculated due to the abscence of laboratory parameters were excluded from the study.

Statistical Analysis

Statistical analyses of the study were performed with SPSS 25.0 (IBM Incorp, IL, USA) program. Descriptive measures were presented as mean±SD or median (Q1-Q3) for numerical measurements according to their normality, and frequency (percentage ratio) for categorical measurements. The normality of numerical measurements was analyzed with the Kolmogorov-Smirnov test. Independent group comparisons were performed by using the Student's t-test and the Mann-Whitney U test. Chi-square test was used to determine the relationships between the categorical variables, distribution-appropriate correlation analyzes were used to determine the relationships between numerical measurements. To determine the factors that affect the severity of PAD, univariate and multivariate logistic regression models were established. The goodness-of-fit values and significance of the model were calculated. The model was created by using the forward likelihood ratio logistic regression method for avoiding the multicollinearity problem. In order to determine the diagnostic features of the TyG index, ROC analysis was performed and diagnostic rates were calculated. A $p<0.05$ value was considered statistically significant by taking the type-I error rate as 5% throughout the study.

The power analysis of the study was performed with the GPower 9.1.2 (Universitaet Kiel, Germany) program. By calculating the triglyceride and glucose values and TgG index of the patients that were selected for the pilot study, the effect size was calculated for mild to moderate and severe patient groups ($d=0.742$). The sample size for each group was calculated as $n=54$ for the power value of 95% and margin of error of 5%. However, the moderate:severe ratio was taken as 1:2, since the number of patients with severity was observed to be higher among the patients admitted to the hospital.

RESULTS

Two hundred patients diagnosed with PAD that underwent peripheral arterial angiography were included in the study. Patients were divided into two groups for the severity of their lesions according to GLASS classification as Group 1 (moderate- G1) and Group 2 (severe- G2), (Table 2).

There was no difference for mean age, gender and comorbidities between the study groups. While chronic kidney disease (CKD) was found to be higher in the G2, CAD was found to be slightly higher in G1. Lower extremity amputation rates were found significantly higher in G2 (Table 3).

Patients were also evaluated according to the Rutherford classificiation and stastistically significant association with GLASS classification was observed ($p<0.001$). Statistically significant relationship was also found between the PAD severity determined by GLASS classification and ABI ($p<0.001$).

TyG values showed statistically significant difference between the groups. TyG was higher in severe group (G2) compared to the moderate group (G1) ($p=0.04$). When TyG comparison was studied for Rutherford classification among study groups (Table 2), TyG was observed significant higher in severe group compared to the moderate group ($p=0.012$).

There was no difference observed for serum total cholesterol, high density lipoprotein (HDL), and low density lipoprotein (LDL) values among the moderate and severe groups. The results of biochemical parameters were summarised in Table 4.

Since the TyG and PAD severity association was found significant in study groups, ROC analysis was applied to determine the index diagnostic value for PAD severity. A significant but low-level ROC curve was obtained. Diagnostic ratios were calculated as 65.5% sensitivity and 58.9% specificity (Fig. 1).

In order to determine the diagnostic factors effecting G2, a binary logistic regression model was created by taking G1 as the reference group. Demographic variables (age and gender), TyG, HDL, LDL and total cholesterol values were included in the model. Variables that could cause multicollinearity problem were excluded. The model was created by using the forward stepwise logistic regression method. The model was found significant (Omnibus $X^2=6.971$; $p=0.008$ and Hosmer-Lemeshow $X^2=10.03$; $p=0.262$). Goodness of fit was found at medium-level (Nagelkerke $R^2=0.05$). Only TyG was found to have a significant effect on the model as a diagnostic factor (OR=2.075), (Table 5).

DİSCUSSİON

In our study, we aimed to investigate the relationship between the severity of PAD, which is determined by using the GLASS classification, and the TyG. According to the best of our knowledge, our study is the first to examine the association of TyG with the severity of PAD determined by using this novel classification system; and similar to other few studies reported in the literature, we have found a significant relationship between TyG and PAD severity. Since we aimed

Table 2
Study groups divided according to GLASS and Rutherford classification.

Group 1 (Moderate)	Group 2 (Severe)
Femoropopliteal 0-2/Aorta-iliac 1	Femoropopliteal 3-4/Aorta-iliac 2
Infrapopliteal 0-2	Infrapopliteal 3-4
Pedal 0	Pedal 1-2
Rutherford Class 0-2	Rutherford Class 3-6

Table 3
Demographic spesifications and comorbidities.

		Group1 N=58 (28.3)	Group2 N=142 (71.1)	Total N=200	
Specifications	Cathegories	N (%)	N (%)	N (%)	*p*
Gender	Female	4 (6.9)	23 (16.2)	27 (13.6)	*0.095*
	Male	54 (93.1)	119 (83.8)	171 (86.4)	
Diabetes Mellitus	none	26 (44.8)	65 (45.8)	90 (45.5)	*0.885*
	Yes	32 (55.2)	77 (54.2)	108 (54.5)	
Hypertension	None	16 (27.6)	44 (31)	59 (29.8)	*0.561*
	Yes	42 (72.4)	98 (69)	139 (70.2)	
Smoker	None	37 (63.8)	98 (69)	133 (67.2)	*0.379*
	Yes	21 (36.2)	44 (31)	65 (32.8)	
Coronary Artery Disease	None	31 (53.4)	89 (63.1)	119 (60.1)	*0.216*
	Yes	27 (46.6)	52 (36.9)	78 (39.4)	
Chronic Renal Failure	None	54 (93.1)	127 (89.4)	179 (90.4)	*0.462*
	Yes	4 (6.9)	15 (10.6)	19 (9.6)	
Repeated Intervention	None	48 (82.8)	127 (89.4)	173 (87.4)	*0.164*
	Yes	10 (17.2)	15 (10.6)	25 (12.6)	
Amputation	None	52 (89.7)	107 (75.4)	159 (79.5)	*0.023**
	Yes	6 (10.3)	35 (24.6)	41 (20.5)	
Rutherford Classification	0+	5 (8.8)	0	5 (2.5)	
	1+	25 (43.9)	3 (2.1)	28 (14.1)	
	2+	21 (36.8)	4 (2.8)	25 (12.6)	
	3+	2 (3.5)	84 (59.2)	86 (43.2)	*<0.001**
	4+	1 (1.8)	41 (28.9)	42 (21.1)	
	5	3 (5.3)	8 (5.6)	11 (5.5)	
	6	0	2 (1.4)	2 (1.0)	
Age (years)	67.09±9.22	68.47±11.42			*0.207*
ABI	0.67±0.13 0.77; 0.69-0.82	0.31±0.12 0.28; 0.24-0.36			*<0.001**

*: Significant at the 0.05 level according to the Chi-Square test; +: the related Rutherford class is significantly different between the groups. ABI: Ankle-Brachial Index.

to determine this association by using novel GLASS classification, we also evaluated the GLASS classification system to understand its relationship with the PAD severity. For this purpose, we compared this system with the Rutherford classification and ABI measurements and found significant agreement between the GLASS and PAD severity.

There has been some studies that investigated multiple pathological consequences of atherosclerosis; however, PAD has been paid less attention than the other pathologies like CAD or stroke [27]. Based on the latest reports, it is estimated that 5.56% ratio of people worldwide aged 25 years and older had PAD [28]. But only 10% of PAD patients

Table 4
Biochemical parameters.

	Group1 (N=58)	Group2 (N=142)	
	Avarage±SS Median; Q1-Q3	Avarage±SS Median; Q1-Q3	*p*
Triglyceride (mg/dL)	135.33±54.29 129; 88.75-161.86	172.28±84.31 153.5; 113-210	*0.040**
Fasting Blood Glucose	132.86±45.62 116.5; 95.25-165	137.56±52.64 126.5; 94.75-173.5	*0.269*
LDL	105.22±35.39 113.5; 75.25-130.75	116.08±37.31 111.14; 88-140	*0.875*
VLDL	27.43±11.21 26; 18.25-32.24	34.03±16.42 30; 22-41.25	*0.061*
HDL	42.9±16.02 40; 34-46.75	41.73±13.53 40; 33-48	*0.907*
Total Cholesterol	170.81±38.16 174.5; 145.5-199.25	188.87±48.97 181.5; 156-220.25	*0.541*
TyG	8.96±0.54 8.9; 8.69-9.41	9.21±0.61 9.2; 8.78-9.49	*0.040**

*: Significant at the 0.05 level according to the Mann-Whitney U test.
TyG: Triglyceride-Glucose Index; LDL: Low density Lipoprotein; HDL:High density Lipoprotein; VLDL: Very Low Density Lipoprotein,

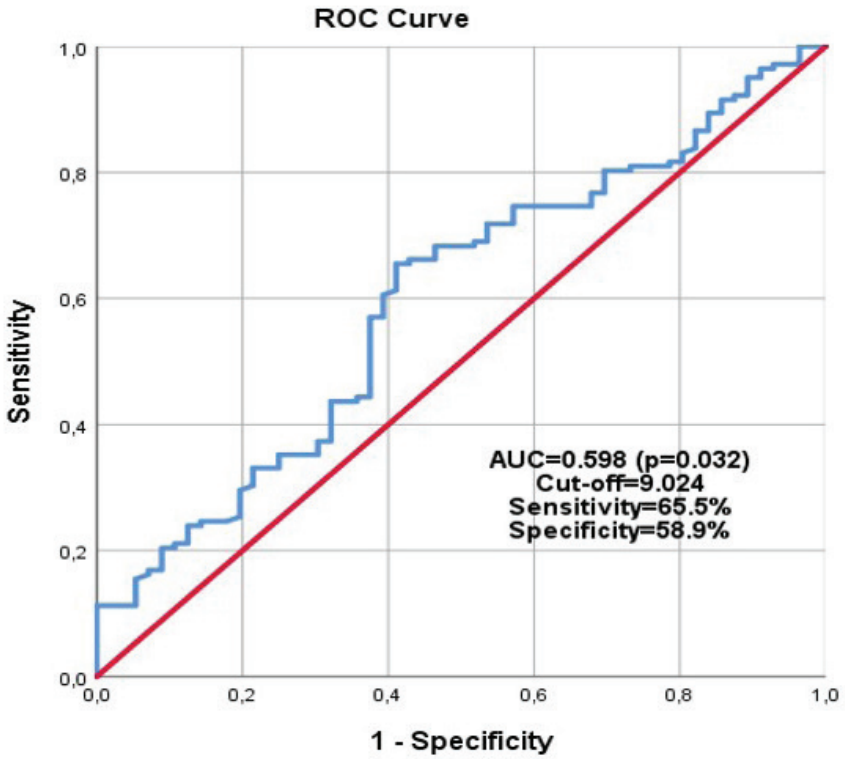

Fig. 1. Triglyceride-Glucose Index ROC curve for patients with severe lesion.

Table 5
Diagnostic factors effective on patients with severe lesion.

Factors	Beta	p	OR	%95 CI
Age	1.528	0.216		
Gender	3.002	0.083		
TyG	0.730	0.011*	2.075	1.183-6.640
LDL	2.264	0.132		
HDL	0.017	0.896		
T. cholesterol	3.193	0.074		

TyG: Triglyceride-Glucose Index; LDL: Low density Lipoprotein; HDL:High density Lipoprotein; T.Cholesterol: Total Cholesterol. *: Significant at the 0.05 level according to the Binary logistic regression analysis.

demonstrate typical symptomatology and the others remain undiagnosed [5]. Therefore, it is important to determine the appropriate biomarkers for the PAD risk and its severity. With regular measurement of the levels of these biomarkers will be important in terms of determining the risk of PAD or monitoring the course of the diagnosed disease. By this means, taking early measures could prevent the progression of the disease at early clinical stages.

Insulin is a hormone that regulates the cell metabolism, and IR is characterized by a deficit in insulin uptake by peripheral tissues. This resistance impairs glucose uptake and glycogen synthesis of tissues and creates an imbalance in lipid oxidation. As glucose homeostasis deteriorates, insulin secretion increases. Secondary to hyperinsulinemia, oxidative stress and an increase in inflammatory responses occur [29,30]. Endothelial cells get affected by this oxidative stress, endothelial function gets impaired and atherosclerosis develops in the chronic period [31]. For these reasons, IR has been seen as an important risk factor for CVD [32,33]. It has been shown that IR and hyperinsulinemia are associated with the development of HT, dyslipidemia and atherosclerosis [34,35]. But data that have reported association of IR and PAD are limited [36,37]. A cross-sectional study of 3242 adults from data in the National Health and Nutrition Examination Survey identified a positive relation between IR and PAD [26,37]. A study with 4208 participants over the age of 65 years in the Cardiovascular Health Study, found that IR was associated with a higher risk of clinical PAD [27,38]. In some reports, TyG was defined as a marker with high specifity and sensitivity for IR [11,38].

The positive relationship of TyG with CVD and atherosclerosis has been shown in many studies [39-41]. Li *et al*. reported in a retrospective study that the TyG could be used as a high risk predictor for CVD [42]. In a study conducted with 5014 healthy individuals, high levels of the TyG were shown to be associated with an increased risk of CVD [41]. In another study involving 4319 patients, a significant association of the TyG with the presence of coronary calcification was reported [39]. IR and PAD association was reported in some studies [27,36,37]. Although there are studies on the association of the TyG with coronary and carotid diseases, studies that show the relationship of the TyG and PAD severity is rare. Chiu et al. reported a significant association between the TyG and low ABI in their study [43]. Kim *et al.*, on the other hand, found that the TyG was associated with arterial stiffness and coronary artery calcification in Korean adults [44]. Among these studies, the study conducted by Duran Karaduman *et al*. showed a significant relationship between the TyG elevation and PAD severity [3]. In addition, there are studies that have investigated the predictability of the TyG for critical limb ischemia [45].

Despite all these reports, studies that examine the severity of the PAD and TyG association according to the anatomical classification systems developed for PAD, such as TASC, are very limited. In the study conducted by Duran Karaduman et al, the relationship between the TyG and PAD complexity and severity was investigated by using TASC classification [3]. In our study we found a statistically significant correlation between TyG and the severity and complexity of PAD detected with GLASS classification ($p=0.04$) similar to the findings of the reports on the association of TyG and PAD severity determined with other angiographic classification systems like TASC [3]. We also compared this novel system with Rutherford system and ABI measurements to determine the positive association of PAD severity and this system. We observed a statistically significant association ($p< 0.01$), (Table 3).

Serum lipids also play an important role in developing atherosclerosis. LDL is the best known parameter for this risk but the relationship between the TGs, CVD and atherosclerosis is still controversial. Recent studies have provided evidence on the fact that TG and TG-rich lipoproteins are among the causes of CVD [10]. It has been shown that the simultaneous presence of hypertriglyceridemia (HTG) promotes the formation of high atherogenic small dense LDL particles [46]. As a summary, there is a relation between TG levels and atherosclerosis [47,48]. In our study, TG levels were also found significantly higher in G2 ($p=0.04$).

There were some limitations of our study. The low number of patients was one of the most important limiting factor. It was mostly due to the limited number of interventional procedures performed in our clinic. In addition, the laboratory parameters that were used in the calculation of TyG were absent in the data of some patients; therefore, the index could not be calculated and these patients were compulsorily excluded from the study. This was another factor that reduced our case number. We think that the low diagnostic value of TyG detected by ROC analysis could increase if the study could be performed with more patients, and by this means the study could become more valuable.

TyG is an easily calculable index. In our study we found a significant relationship between the severity of PAD and TyG, which was determined by using the novel GLASS classification, similar to previous studies which use other anatomic classification systems. In the light of these findings, we think that this index would be a useful and simple marker for detecting the patients' disease severity for newly diagnosed or cases has been treated with medically or other invasive methods. Possible early detection of worse onset or worsening of the diagnosed disease can be predicted by routine use of this parameter, and morbidities could be prevented by applying appropriate treatments to these patients in early periods. But more large scale studies are needed to support this conclusion.

Funding

Authors declare no funding for this article.

Conflict of Interest

Authors declare no conflicts of interests

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Invest Clin 63(4): 376 - 387, 2022 https://doi.org/10.54817/IC.v63n4a05

Capacidad pro-apoptótica *in vitro* de *Valeriana rígida* y *Valeriana decussata* sobre una línea celular de cáncer de mama.

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Palabras clave: Bcl-2, BAX; extracto plantas; *Valeriana;* cáncer de mama.

Resumen. El cáncer representa un problema de salud pública a nivel mundial, con altas tasas de incidencia y mortalidad en países desarrollados y no desarrollados. En la actualidad se están evaluando alternativas terapéuticas de origen natural, con el propósito de establecer tratamientos más eficientes y menos invasivos. Dado que la apoptosis es el tipo de muerte programada que experimentan las células cancerosas por los tratamientos con los fármacos antineoplásicos,el objetivo de esta investigación, fue evaluar *in vitro* la capacidad pro-apoptótica y citotóxica de los extractos de valeriana, sobre una línea celular de cáncer de mama (MCF-7). En este estudio las células MCF7 se cultivaron y trataron con diferentes concentraciones de los extractos de la raíz, hojas y tallos de *Valeriana rígida* y *Valeriana decussata.* La viabilidad celular se evaluó mediante el ensayo MTT. Para la determinación de la expresión génica de las proteínas anti y pro-apoptóticas (Bax, Bcl-2 y p53), se usó el ensayo de la PCR cuantitativa de transcripción inversa. Las diferentes concentraciones de los extractos (10^{-8} a 10^{-1} mg/mL) disminuyeron la viabilidad (proliferación) celular en concentraciones dependientes. Estos extractos indujeron la expresión génica de las proteínas Bax y Bcl-2, pero no de p53. La expresión de Bax fue mayor que la de Bcl-2 e indujo un elevado índice Bax/Bcl-2 (condición pro-apoptotica). En conclusión, se determinó que los extractos de *Valeriana decussata* y *Valeriana rígida* poseen efecto reductor de la viabilidad (proliferación) de la línea celular de cáncer de mama MCF-7, probablemente mediado por la alteración de la relación de las proteínas Bax y Bcl-2 vinculadas a la apoptosis.

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***In vitro* pro-apoptotic capacity of *Valerian rigid* and *Valerian decussata* in cancer cells.**

Invest Clin 2022; 63 (4): 376 – 387

Keywords: Bcl-2; BAX; plant extract; *Valeriana;* breast cancer.

Abstract. Cancer represents a worldwide public health problem, with high incidence and mortality rates in developed and undeveloped countries. Currently, therapeutic alternatives of natural origin are being evaluated with the purpose of establishing more efficient and less invasive treatments. Apoptosis is the type of programmed death cancer cells undergo during treatment with anti-neoplastic drugs. Therefore, the aim of this research was to evaluate *in vitro* the pro-apoptotic and cytotoxic capacity of valerian extracts on a breast cancer cell line (MCF-7). In this study, MCF7 cells were cultured and treated with different concentrations of the extracts of the root, leaves and stems of *Valeriana rígida and Valeriana decussata*. Cell viability was assessed by the MTT assay. Quantitative reverse transcription PCR assays were used for the determination of gene expression of anti- and proapoptotic proteins (Bax, Bcl-2, p53). Different concentrations of the extracts (10^{-8} to 10^{-1} mg/mL) decreased cell viability (proliferation) in a concentration-dependent manner. These extracts induced gene expression of Bax and Bcl-2 proteins but not of p53. The expression of Bax was higher than that of Bcl-2, causing an elevated Bax/Bcl-2 ratio (proapoptotic condition). In conclusion, it was determined that *Valeriana decussata and Valeriana rígida* extracts have a viability (proliferation) reducing effect on the MCF-7 breast cancer cell line, probably mediated by altering the ratio of Bax and Bcl-2 proteins linked to apoptosis.

Recibido: 21-04-2022 Aceptado: 16-07-2022

INTRODUCCIÓN

El cáncer se define como el crecimiento anormal del tejido, debido a la proliferación incontrolada de células anormales o mutantes [1]. Es una enfermedad que representa un problema de salud pública, con una alta incidencia de muerte en todo el mundo [2]. Uno de los principales mecanismos de control en los organismos, es la muerte celular programada; proceso genéticamente regulado, en el cual las células deben morir para preservar el desarrollo, homeostasis e integridad de los organismos multicelulares [3]. Entre estos mecanismos se encuentra la apoptosis, que se caracteriza por ser un proceso controlado por la velocidad de división celular, además de reducir la proliferación de células malignas [4].

Existen diferentes tipos de tratamiento contra el cáncer que han sido estudiados y representan a los agentes quimioterapéuticos que permiten el control y la remisión [5]. Sin embargo, estos agentes promueven la aparición de efectos secundarios a los procedimientos oncológicos y que inciden directamente en la calidad de vida de los pacientes [6].

A nivel mundial las plantas representan una gran alternativa, que sienta las bases del estudio de sus beneficios en la medicina actual. Durante siglos han sido empleadas con

diferentes propósitos médicos, entre ellos el tratamiento del cáncer. La Organización Mundial de la Salud (OMS), reconoce los beneficios del uso de plantas en Medicina por sus efectos farmacológicos [7]. Se han aislado diferentes moléculas a partir de ellas, para la elaboración de fármacos quimioterapéuticos, que han demostrado una alta eficacia clínica y potencial anticancerígeno.

La palabra valeriana designa a cualquier planta del género Valeriana, el cual pertenece a la familia Caprifoliaceae y cuya especie más conocida es *V. officinales* [8]. Es una planta originaria de Europa y partes de Asia. Se conocen alrededor de 400 especies distribuidas en diferentes lugares del mundo, entre ellos Ecuador. Su morfología es muy variable, forman fuertes rizomas con sus raíces alargadas y pequeñas ramificaciones y se caracterizan por emitir un olor fuerte y desagradable. Se le han conferido diferentes propiedades medicinales debido a su composición química (valtrato, flavonoides, aceites como valeranona y valeranal, monoterpenos bicíclicos y ácidos valéricos), y es comúnmente usada para tratar afecciones médicas como el insomnio, el estrés, enfermedades neurológicas y la ansiedad [9].

El valtrato es considerado un éster epoxi iridoide, aislado de la medicina herbal china, especialmente de la *Valeriana jatamansi* Jones, y se le ha conferido actividad antiproliferativa contra diversas líneas celulares de cáncer humano; sin embargo, los mecanismos moleculares implícitos no se conocen con exactitud [10].

En la actualidad, se han reportado investigaciones en las cuales se han incluido plantas de valeriana, se usaron semillas, rizomas, raíces, estolones, extractos, aceites esenciales, resinas y otros componentes, para el estudio de las vías apoptóticas y los efectos antitumorales que estas especies pudiesen inducir [11].

Los ácidos linolénicos conjugados (CLN), obtenidos de los aceites de semillas de *V. officinalis,* demostraron que podrían ser fácilmente absorbidos por las células cancerosas como ácidos grasos libres, mostrando un buen potencial como sustancias antitumorales [12].

De igual forma, se ha probado el efecto de los extractos de valerianas en algunos tipos de cáncer como el de hígado [13], pancreático [14], cervical [15] y de mama. Shi y col. [16] demostraron que el ácido valérico obtenido de raíces de *V. officinalis,* puede disminuir la proliferación de células de cáncer de mama, mediando modificaciones epigenéticas como la inhibición de las histonas desacetilasas (HDAC) y alteraciones en la metilación del ADN. Estas propiedades del ácido valérico en la HDAC también tienen un amplio espectro de actividad anticancerígena, con una alta citotoxicidad para el cáncer de hígado en ensayos de proliferación celular, formación de colonias, cicatrización de heridas, invasión celular y formación de esferoides en 3D. Algunos modelos de ratón demostraron que la administración sistemática de ácido valérico encapsulado en nanopartículas a base de lípidos, disminuye significativamente la carga tumoral, mejorando la tasa de supervivencia [13].

Se ha descrito ampliamente el proceso de la apoptosis y su función biológica en la patogenia de varias enfermedades tales como el cáncer, trastornos metabólicos, neuropatías, lesiones miocárdicas y alteraciones del sistema inmunitario. Tomando como premisa que aproximadamente el 50% de los tumores humanos están asociados a mutaciones del gen p53, es de vital importancia el estudio de los mecanismos de apoptosis en el proceso de carcinogénesis, así como el efecto de principios activos en estos mecanismos, en pro de la identificación de posibles blancos terapéuticos. Las señales de apoptosis están reguladas por diferencias en la expresión de proteínas promotoras e inhibidoras, especialmente los miembros de la familia Bcl-2 que están integrados por: Bcl-2, Bax, Bad, Bcl-X1, Bcl-Xs, Mcl-1 y algunas proteínas efectoras como las caspasas. Es necesario conocer la expresión de proteínas y su incremento y disminución a expensas de

posibles tratamientos que puedan ser coadyuvantes en el proceso de muerte celular en células cancerígenas.

En virtud que existen diversos mecanismos por dilucidar y tomando en consideración que se han reportado otras especies de valeriana propias de la región, que pudiesen estudiarse en modelos celulares, el presente estudio planteó como objetivo determinar el potencial terapéutico *in vitro* de *Valeriana rígida* y *Valeriana decussata* en células MCF-7, mediante la determinación de la capacidad pro-apoptótica y citotóxica del extracto de sus hojas, tallos y raíces.

MATERIAL Y MÉTODOS

Preparación de extractos

Los especímenes de valerianas se recolectaron en el cerro Igualata, ubicado en el cantón Quero, perteneciente a la provincia de Tungurahua-Ecuador, previa autorización emitida por la Dirección Provincial del Ambiente Tungurahua N°06-2018-IC-FLO-FAU-DPAT-VS. A cada una de las especies se les evaluó las características organolépticas y físicas; como estado, olor y estructura de hojas, tallos, flores y raíces. El material vegetal recolectado fue transportado al laboratorio de la Facultad de Ciencias e Ingeniería en Alimentos y Biotecnología para llevar a cabo el proceso de lavado y secado a 60°C. Luego se pulverizaron, tamizaron y se almacenaron a temperatura ambiente hasta su uso posterior. Para la obtención del extracto, se resuspendió el material vegetal pulverizado en PBS 1X estéril relación 1:20, después se homogenizó con un vórtex. Posteriormente, cada extracto fue transferido a otro tubo estéril de 15 mL a través de un filtro de 0,22 μm, con el objetivo de eliminar contaminantes o material seco y así conseguir la esterilidad. Se obtuvo un extracto de tipo acuoso con PBS en concentración 1X en relación 1:20 con los extractos de Valeriana, los cuales presentaron una coloración marrón de poca viscosidad. Se escogió este tipo de extracto debido a la composición bioquímica de las valerianas que, a la habitual administración farmacológica y el PBS como solución tampón, mantiene la presión osmótica y el equilibrio de las células. Todos los extractos se mantuvieron en congelación (-20°C).

Cultivo Celular

Para el desarrollo de la investigación se empleó la línea celular MCF7 ATCC®HTB-22™ derivada del cáncer de mama humano. Las células se cultivaron en frascos Roux de 75mL, en medio DMEN (Dulbeccos Modified Eagle Medium), suplementado con 10% de suero fetal bovino (FBS), 1% de penicilina/estreptomicina (100 μg/mL) y 1% de glutamina. Las células fueron incubadas a 37°C, 5% de CO_2 y atmosfera húmeda. Los pases celulares se realizaron en base a la proliferación y población celular contenida en los frascos, con una confluencia entre el 80 y 100%. Para el contaje celular se utilizó una cámara de Neubauer siguiendo el protocolo establecido, a la cual se le añadieron 10 μL de la disolución final teñida con "azul tripán" y se leyó en un microscopio óptico (magnificación 10X). Finalmente, se obtuvo el número de células viables por cada mL de suspensión.

Análisis de viabilidad celular

Para calcular la citotoxicidad de los extractos en células MCF-7, se empleó el ensayo MTT (3- (4,5-dimetiltiazol-2-il) -2,5-difeniltetrazolio) de Invitrogen™ (USA). El tratamiento con los extractos se realizó a diferentes concentraciones en diluciones seriadas, en función de la base logarítmica 10 (Rango 10^{-8} a 10^{-1} de acuerdo con la concentración inicial de proteínas contenidas en cada extracto: *decussata* raíz (DDR) (4mg/mL), *decussata* hoja y tallo (DHT) (6,3 mg/mL), *rígida* hoja y tallo (RHT) (3,6 mg/mL) y *rígida* raíz (RR) (3,2 mg/mL). Seguidamente se agregaron los extractos en las células cultivadas en placas de 96 pocillos con una densidad de 1,0 x 10^4 cel/pozo y se leyeron en un lector de placas Perkin Elmer Víctor X3 (USA), según el protocolo descrito por

la casa comercial [17]. Todos los experimentos se realizaron por duplicado. El valor de la concentración inhibitoria media (IC50) se determinó utilizando el programa GraphPad Prism V7.0 (Software Inc., San Diego, CA, USA).

Determinación de Proteínas

Para la cuantificación de proteínas se empleó el método Bradford, se elaboró la curva de calibración con albúmina de suero bovino (BSA) a 0,25, 0,50, 0,75, 1,00, 1,25, 1,50 y 1,75 mg/mL. se realizó la lectura de las absorbancias a 595 nm en un espectrofotómetro de microplacas (PerkinElmer Víctor X3, USA).

Expresión Génica

Para comparar los datos experimentales, se realizó una RT-qPCR para determinar la expresión de Bax, Bcl-2 y p53 a partir del ARN obtenido de las células MCF-7 tratadas y del control (no tratadas).

El proceso se inició con la extracción de ARN de la suspensión celular, utilizando el estuche PureLink® RNA Mini Kit, según el protocolo del fabricante [18], se determinó el valor de concentración (ng/μL) y se almacenó a -80°C (12)**.** El ADNc se sintetizó a partir del ARN total utilizando el protocolo Maxima First Strand cDNA Synthesis (ThermoScientific) [19].

Finalmente, para realizar la RT-qPCR se utilizó el protocolo GoTaq® qPCRMaster. En una tira de tubos Eppendorf se agregaron 10 μL de Mix (2X), 1 μL de Forward Primer, 1 μL de Reverse Primer (Tabla 1), 1,6 μL del templado de ADNc por cada tubo (según la reacción) y se completó hasta 20 μL con agua libre de ARNasa. Los tubos se centrifugaron y fueron colocados en el Termal Cycler (CFX96** Real-time System, USA). El equipo se programó según el protocolo de Promega (Promega GoTaq®, 2014). Las condiciones de amplificación fueron: activación de la polimerasa a temperatura de 95°C durante 30 segundos, desnaturalización del ADNc a 95°C durante 15 segundos; amplificación a 58°C durante 25 segundos en un total de 32 ciclos.

Análisis estadístico

Los resultados obtenidos se analizaron con el programa GraphPadPrism V7.0 (Software Inc., San Diego, CA, USA). Se expresaron como media y desviación estándar ($\bar{x}\pm$ DS). Para el análisis de varianza se utilizó ANOVA de un solo factor con post test de Bonferroni, considerando significativos los valores de $p \leq 0,05$ con un 95% de límite de confianza.

RESULTADOS

Efecto de los extractos en la viabilidad de las células neoplásicas

La Fig. 1 muestra el efecto de los extractos de DR y DHT en la viabilidad de las células neoplásicas. Si bien se observaron diferentes grados de efecto inhibitorio según las dosis de los extractos, el mayor efecto inhibitorio se encontró en la dosis de 10^{-3} mg/mL. La Fig. 2 muestra el efecto inhibitorio de los extractos de la RR y la RHT. La RHT

Tabla 1
Secuencia de Primers.

Gen	Forward Primer	Reverse Primer
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGA
ACTINA	CGGTTCCGATGCCCTGAGGCTCTT	CGTCACACTTCATTGATGGAATTGA
BAX	CAAGACCAGGGTGGTTGGG	ATCTTTGTGGCGGGAGTG
Bcl-2	CATGTGTGTGGAGAGCGTCAA	GCCGGTTCAGGTACTCAGTCA
p53	GACGGTGACACGCTTCCCTGGATT	GGGAACAAGAAGTGGAGAATGTCA

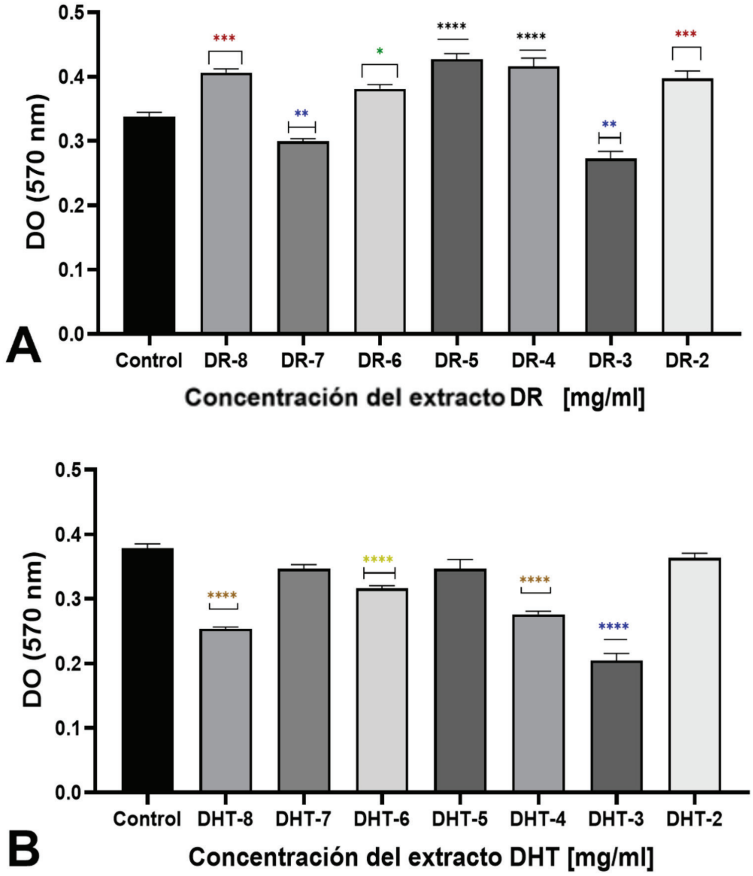

Fig. 1. Ensayo de viabilidad de los extractos de valeriana en células MCF-7. Las células se sembraron a $1x10^4$ células por pozo y se trataron con dosis crecientes (10^{-8} mg/mL a 10^{-1}mg/mL) de *Valeriana decussata* raiz (**DR:** 4mg/mL) (A) y *Valeriana decussata* hojas y tallos (**DHT:** 6,3 mg/mL) (B). Los experimentos se realizaron por triplicado para evaluar la concentración inhibitoria media máxima (IC50: 10^{-3} mg/mL) para los extractos de plantas. Tratamiento Vs. control (α= 0,05, *P=<0.05; **P=<0.01; ***P<0.001; ****P<0,0001).

presentó fluctuaciones en el efecto inhibitorio de acuerdo con las dosis (Fig. 2A), sin embargo, todas las dosis de RR fueron similarmente inhibitorias (Fig. 2B). Ambos extractos tuvieron el mayor efecto inhibitorio en la dosis de 10^{-3} mg/mL.

Expresión génica de proteínas vinculadas con la apoptosis

No se observó expresión génica de la proteína p53 en las células tratadas con los extractos, sin embargo y en menor proporción a las células no tratadas, se apreció expresión de Bax y Bcl-2 en las células neoplásicas tratadas, encontrándose mayor expresión de Bax (Figs. 3 A y B). Como es esperado índice Bax/Bcl-2 se observa elevado en los diferentes tratamientos con los extractos, presentándose los mayores valores en los tratamientos con extractos de *Valeriana decussata* (HT) y *Valeriana rígida* (RR) (Fig. 3C).

El gen de control endógeno GAPDH no presentó variación significativa en su expresión génica después de la aplicación de los tratamientos de *Valeriana decussata* y *Valeriana rígida* (Fig. 4). La expresión relativa normalizada de la proteína Bcl-2 y Bax se muestran en la (Fig. 4).

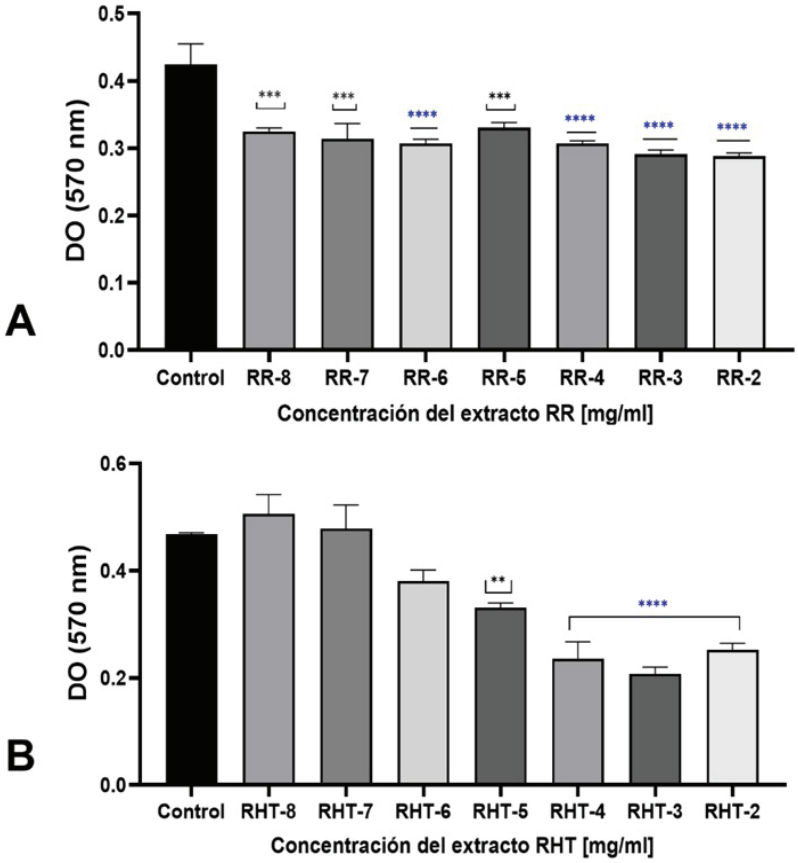

Fig. 2. Ensayo de viabilidad de los extractos de valeriana en células MCF-7. Las células se sembraron a $1x10^4$ celulas por pozo y se trataron con dosis crecientes (10^{-11} mg/mL a 10^{-1} mg/mL.) de Valeriana rígida raíz (RR: 3,2 mg/mL) (A) y Valeriana rígida hojas y tallos (RHT: 3,6 mg/mL) (B). Los experimentos se realizaron por triplicado para evaluar la concentración inhibitoria media máxima (IC50: 10-3 mg/mL) para los extractos de plantas. Tratamiento Vs. control (α= 0,05,**P<0,05; ****P<0,0001).

DISCUSIÓN

Es de gran importancia el estudio de compuestos fitoquímicos en pro de la búsqueda de alternativas o coadyuvantes en la terapéutica convencional del cáncer. A la fecha se han atribuido múltiples propiedades a las valerianas principalmente a la *V. officinalis*; sin embargo, son muchas las variedades que quedan por estudiar, algunas endémicas de la Sierra del Ecuador.

En la actualidad, no se conoce un efecto real de la Valeriana sobre células cancerígenas, ya que su uso terapéutico se ha limitado al sistema nervioso para el tratamiento del insomnio y la ansiedad. Sin embargo, la Valeriana tiene compuestos que potencian la interacción sinérgica de otros medicamentos depresores del SNC (sistema nervioso central) al inducir la eficacia del tratamiento [21]. También existen investigaciones de la inducción de la actividad ansiolítica, la relación con el efecto del ácido valórico y la interferencia del ácido acetoxi valerénico. Garrido en el 2007 determinó mediante la técnica de cromatografía de gases, que la *Valeriana Prionophylla* contiene ácido valerénico, acetoxivalerénico e hidroxivalerénico y los Valepotriatos contienen derivados como el baldrinal, homobaldrinal, acevaltrato, didrovaltrato, didrovaltrato, isovaltraro y valtrato. La cantidad de compuestos y ácidos

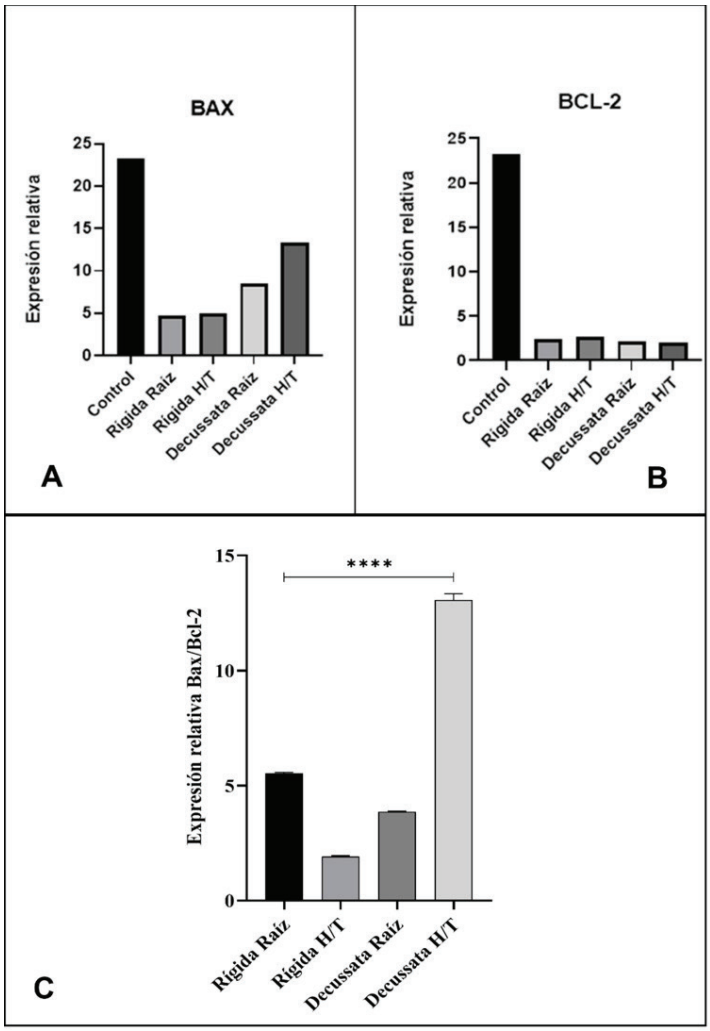

Fig. 3. Expresión relativa normalizada de las proteínas Bax (A) y Bcl-2 (B) según el método ΔΔCt: Se compara la expresión relativa del gen endógeno GAPDH vs la expresión génica de las células MCF-7. La expresión relativa Bax/Bcl-2 se muestra en el panel C (****P≤0,0005), (***P≤0,005), (**P≤0,05).

tuvo diferencias en función de los extractos a partir de hojas y raíces [22].

El ensayo de MTT evidenció un potencial efecto de los extractos en la proliferación celular y el bajo nivel de citotoxicidad, mostrando el comportamiento y funcionabilidad de las células MCF-7 sometidas a tratamientos [23].

Los extractos inhiben la proliferación celular de forma dependiente a una concentración determinada en la dilución logarítmica 10^{-3} [24]. Al comparar el comportamiento celular con cada extracto se evidenció que para ambas valerianas se obtuvieron mejores resultados de los extractos a partir de hojas y tallos. Esto puede ser ocasionado por composición bioquímica presente en las plantas que inducen a la inhibición en la proliferación celular.

La proliferación evidenciada en concentraciones logarítmicas diferentes a 10^{-3} posiblemente pudo ser ocasionada por la interacción entre las células y el extracto acuoso como lo mencionaron Flores y Martínez en 2019 [25] dependiendo del tipo de planta y el extracto acuoso que se utilice, la prolifera-

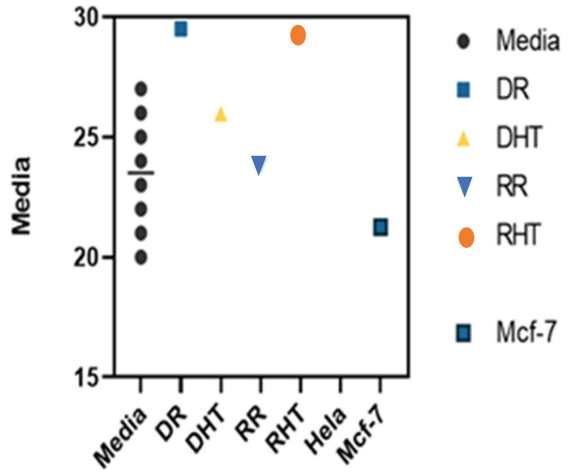

Fig 4. Análisis de los valores de Cts obtenidos para el gen GAPDH: Se analizó y validó al GAPDH como gen calibrador y de referencia. (P>0,05) Las medias de las muestras estuvieron en el margen de la media para GAPDH (P>0,05). *Valeriana decussata* raiz **(DR)** *Valeriana decussata* hojas y tallos **(DHT)** *Valeriana rigida* raiz **(RR)** *Valeriana rigida* hojas y tallos **(RHT).**

ción celular variará para diferentes concentraciones.

El análisis de varianza mostró diferencias significativas entre concentraciones, dependientes de las especies de valeriana y la estructura vegetal con la que se preparó el extracto y los ensayos; sin embargo, según otros estudios realizados con valeriana, las porciones subterráneas de la planta contienen mayor concentración de compuestos citotóxicos, lo cual podría explicar que en estas investigaciones la citotoxicidad fue mayor. Es importante mencionar que algunos extractos no presentaron diferencia significativa lo cual se pudo originar por la composición bioquímica de la planta, además que las especies incluidas no han sido estudiadas previamente. Está variación puede ser ocasionada como una respuesta de las células ante sustancias extrañas, que considera como peligrosas para su interior o por compuestos presentes en la valeriana que inducen la proliferación, por lo cual es relevante realizar a futuro ensayos fitoquímicos que muestren la composición los extractos.

Los resultados de la expresión génica mostraron variaciones de las proteínas que inducen la apoptosis; estos cambios pueden ser ocasionados por la composición bioquímica presente en las raíces, hojas y tallos de las Valerianas, de allí la importancia de haber realizado la presente investigación. Sin embargo, es necesario profundizar en la composición química y los grupos funcionales presentes en las diferentes especies de Valeriana que estarían incidiendo directamente en la capacidad pro-apoptótica de las células MCF-7.

Se pudo observar que la expresión de genes pro-apoptóticos Bax y Bcl-2 para RR y DHT no superaron el valor alcanzado por el control, esto es un indicativo de la ausencia de compuestos inductores de dichos genes o la represión de estos. Sin embargo, para las demás muestras, se evidenció un aumento de expresión con relación al control. Los valores bajos de expresión génica pueden ser debidos a varios factores, sin embargo, se cree que están principalmente relacionados con el solvente y el método usados para obtener el extracto. Según Páez-Hernández y col. [26], la mayor cantidad de compuestos con actividad quimiprotectora e inductora de los genes Bax y Bcl-2, se encuentra en los aceites esenciales de las plantas.

La relación de las proteínas Bcl-2 y Bax es muy importante en la activación de la apoptosis ya que esta depende de la regulación de ambas. Bcl-2 es una familia de proteínas que presenta actividad anti-apoptótica, que ha generado el estudio de su regulación en la apoptosis y la respuesta celular ante diferentes terapias contra el cáncer. Por su parte, Bcl-2 puede inducir o reprimir la liberación de factores indispensables para la apoptosis como citocromo c y el AIF (Factor inductor de apoptosis) [27]. La proteína pro-apoptótica Bax es una subfamilia homóloga de Bcl-2. Para que se desencadene el proceso de apoptosis es necesaria la regulación de Bax/Bcl-2. Bax tiene como función principal

la permeabilidad de la mitocondria. Estudios realizados por Hussein y Chavi en 2015, demostraron que la inducción de apoptosis en células MCF-7 era ocasionada por la regulación de Bax [28], lo cual está relacionado con la inducción apoptótica de p53 que regula a otras proteínas pro-apoptóticas como Bax. En este estudio se pudo apreciar incremento en la expresión génica de Bax en relación con la expresión de Bcl-2, lo que conduciría a la permeabilización de las mitocondrias con el subsiguiente proceso apoptótico [29].

La proteína p53 no se expresó en las células tratadas con extractos de valeriana, esto pudo ser ocasionado por la interacción de los compuestos presentes en las especies de valeriana que indujeron a la activación de proteínas celulares, que pudieron afectar el funcionamiento de p53. Esto puede ser un indicativo de la proliferación celular a ciertas concentraciones de los extractos, ya que, si no se activa la proteína por daño genético o alteraciones en el mecanismo de control, puede ocasionar la proliferación celular [30].

La proteína p53 cumple diversas funciones biológicas y es un factor indispensable en los procesos intra y extracelulares. En el presente estudio, se evaluó la capacidad de inducción al suicidio celular apoptótico. Diferentes factores de estrés inducen a que la proteína p53 cruce la mitocondria, active la expresión de genes pro-apoptóticos e inhiba la expresión de genes anti-apoptóticos [26]. Un estudio realizado en células MCF-7 con extracto de *Trifolium Pratens L.*, demostró que inducía apoptosis mediante la regulación de la proteína p53, de manera dependiente de la dosis y del tiempo del extracto, induciendo autofagia y apoptosis [31]. En nuestro estudio, la falta de expresión génica de la proteína p53 en las células tratadas con los extractos, sugiere un papel no relevante de esta proteína.

En función de los resultados obtenidos se puede inferir que las diferentes especies de Valeriana estudiadas, contienen compuestos que inducen la expresión de proteínas que dirigen la apoptosis en células cancerígenas de mama. Se requieren más estudios cuantitativos que permitan esclarecer el efecto de estos extractos en otros tipos de cáncer y la verificación cuantitativa del desarrollo de la apoptosis, debido a la expresión de las proteínas pro-apoptóticas como mecanismo de acción de estos sustratos. Este estudio establece la posibilidad del uso de los derivados de la valeriana para optimizar la terapia contra este tipo de cáncer.

AGRADECIMIENTO

A la Universidad Técnica de Ambato, a través de la Dirección de Investigación y Desarrollo (DIDE) por el financiamiento de los proyectos: "Estudio etnobotánico y bioensayos de plantas medicinales relacionadas con el tratamiento de cáncer de cérvix en la ciudad de Ambato, provincia de Tungurahua, Ecuador, aprobado con resolución: 0904-CU-P-201, y "Caracterización morfológica molecular fitoquímica y métodos de propagación en especies silvestres del género valeriana de la provincia de Tungurahua" aprobado con la resolución: 1569-CU-P-2017.

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- Trabajo Experimental YC/JWI/MA.
- Recolección/obtención de resultados YC/JWI/MA.
- Recolección de especies vegetales y preparación del material de estudio JHV/JWI.

• Análisis e interpretación de datos YC/JWI/MA.

• Redacción del manuscrito YC/JWI.

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Conflictos de interés

Los autores declaran no tener ningún conflicto de interés.

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Invest Clin 63(4): 376 - 387, 2022 https://doi.org/10.54817/IC.v63n4a05

Capacidad pro-apoptótica *in vitro* de *Valeriana rígida* y *Valeriana decussata* sobre una línea celular de cáncer de mama.

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Palabras clave: Bcl-2, BAX; extracto plantas; *Valeriana;* cáncer de mama.

Resumen. El cáncer representa un problema de salud pública a nivel mundial, con altas tasas de incidencia y mortalidad en países desarrollados y no desarrollados. En la actualidad se están evaluando alternativas terapéuticas de origen natural, con el propósito de establecer tratamientos más eficientes y menos invasivos. Dado que la apoptosis es el tipo de muerte programada que experimentan las células cancerosas por los tratamientos con los fármacos antineoplásicos,el objetivo de esta investigación, fue evaluar *in vitro* la capacidad pro-apoptótica y citotóxica de los extractos de valeriana, sobre una línea celular de cáncer de mama (MCF-7). En este estudio las células MCF7 se cultivaron y trataron con diferentes concentraciones de los extractos de la raíz, hojas y tallos de *Valeriana rígida* y *Valeriana decussata.* La viabilidad celular se evaluó mediante el ensayo MTT. Para la determinación de la expresión génica de las proteínas anti y pro-apoptóticas (Bax, Bcl-2 y p53), se usó el ensayo de la PCR cuantitativa de transcripción inversa. Las diferentes concentraciones de los extractos (10^{-8} a 10^{-1} mg/mL) disminuyeron la viabilidad (proliferación) celular en concentraciones dependientes. Estos extractos indujeron la expresión génica de las proteínas Bax y Bcl-2, pero no de p53. La expresión de Bax fue mayor que la de Bcl-2 e indujo un elevado índice Bax/Bcl-2 (condición pro-apoptotica). En conclusión, se determinó que los extractos de *Valeriana decussata* y *Valeriana rígida* poseen efecto reductor de la viabilidad (proliferación) de la línea celular de cáncer de mama MCF-7, probablemente mediado por la alteración de la relación de las proteínas Bax y Bcl-2 vinculadas a la apoptosis.

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***In vitro* pro-apoptotic capacity of *Valerian rigid* and *Valerian decussata* in cancer cells.**

Invest Clin 2022; 63 (4): 376 – 387

Keywords: Bcl-2; BAX; plant extract; *Valeriana;* breast cancer.

Abstract. Cancer represents a worldwide public health problem, with high incidence and mortality rates in developed and undeveloped countries. Currently, therapeutic alternatives of natural origin are being evaluated with the purpose of establishing more efficient and less invasive treatments. Apoptosis is the type of programmed death cancer cells undergo during treatment with anti-neoplastic drugs. Therefore, the aim of this research was to evaluate *in vitro* the pro-apoptotic and cytotoxic capacity of valerian extracts on a breast cancer cell line (MCF-7). In this study, MCF7 cells were cultured and treated with different concentrations of the extracts of the root, leaves and stems of *Valeriana rígida and Valeriana decussata*. Cell viability was assessed by the MTT assay. Quantitative reverse transcription PCR assays were used for the determination of gene expression of anti- and proapoptotic proteins (Bax, Bcl-2, p53). Different concentrations of the extracts (10^{-8} to 10^{-1} mg/mL) decreased cell viability (proliferation) in a concentration-dependent manner. These extracts induced gene expression of Bax and Bcl-2 proteins but not of p53. The expression of Bax was higher than that of Bcl-2, causing an elevated Bax/Bcl-2 ratio (proapoptotic condition). In conclusion, it was determined that *Valeriana decussata and Valeriana rígida* extracts have a viability (proliferation) reducing effect on the MCF-7 breast cancer cell line, probably mediated by altering the ratio of Bax and Bcl-2 proteins linked to apoptosis.

Recibido: 21-04-2022 Aceptado: 16-07-2022

INTRODUCCIÓN

El cáncer se define como el crecimiento anormal del tejido, debido a la proliferación incontrolada de células anormales o mutantes [1]. Es una enfermedad que representa un problema de salud pública, con una alta incidencia de muerte en todo el mundo [2]. Uno de los principales mecanismos de control en los organismos, es la muerte celular programada; proceso genéticamente regulado, en el cual las células deben morir para preservar el desarrollo, homeostasis e integridad de los organismos multicelulares [3]. Entre estos mecanismos se encuentra la apoptosis, que se caracteriza por ser un proceso controlado por la velocidad de división celular, además de reducir la proliferación de células malignas [4].

Existen diferentes tipos de tratamiento contra el cáncer que han sido estudiados y representan a los agentes quimioterapéuticos que permiten el control y la remisión [5]. Sin embargo, estos agentes promueven la aparición de efectos secundarios a los procedimientos oncológicos y que inciden directamente en la calidad de vida de los pacientes [6].

A nivel mundial las plantas representan una gran alternativa, que sienta las bases del estudio de sus beneficios en la medicina actual. Durante siglos han sido empleadas con

diferentes propósitos médicos, entre ellos el tratamiento del cáncer. La Organización Mundial de la Salud (OMS), reconoce los beneficios del uso de plantas en Medicina por sus efectos farmacológicos [7]. Se han aislado diferentes moléculas a partir de ellas, para la elaboración de fármacos quimioterapéuticos, que han demostrado una alta eficacia clínica y potencial anticancerígeno.

La palabra valeriana designa a cualquier planta del género Valeriana, el cual pertenece a la familia Caprifoliaceae y cuya especie más conocida es *V. officinales* [8]. Es una planta originaria de Europa y partes de Asia. Se conocen alrededor de 400 especies distribuidas en diferentes lugares del mundo, entre ellos Ecuador. Su morfología es muy variable, forman fuertes rizomas con sus raíces alargadas y pequeñas ramificaciones y se caracterizan por emitir un olor fuerte y desagradable. Se le han conferido diferentes propiedades medicinales debido a su composición química (valtrato, flavonoides, aceites como valeranona y valeranal, monoterpenos bicíclicos y ácidos valéricos), y es comúnmente usada para tratar afecciones médicas como el insomnio, el estrés, enfermedades neurológicas y la ansiedad [9].

El valtrato es considerado un éster epoxi iridoide, aislado de la medicina herbal china, especialmente de la *Valeriana jatamansi* Jones, y se le ha conferido actividad antiproliferativa contra diversas líneas celulares de cáncer humano; sin embargo, los mecanismos moleculares implícitos no se conocen con exactitud [10].

En la actualidad, se han reportado investigaciones en las cuales se han incluido plantas de valeriana, se usaron semillas, rizomas, raíces, estolones, extractos, aceites esenciales, resinas y otros componentes, para el estudio de las vías apoptóticas y los efectos antitumorales que estas especies pudiesen inducir [11].

Los ácidos linolénicos conjugados (CLN), obtenidos de los aceites de semillas de *V. officinalis,* demostraron que podrían ser fácilmente absorbidos por las células cancerosas como ácidos grasos libres, mostrando un buen potencial como sustancias antitumorales [12].

De igual forma, se ha probado el efecto de los extractos de valerianas en algunos tipos de cáncer como el de hígado [13], pancreático [14], cervical [15] y de mama. Shi y col. [16] demostraron que el ácido valérico obtenido de raíces de *V. officinalis,* puede disminuir la proliferación de células de cáncer de mama, mediando modificaciones epigenéticas como la inhibición de las histonas desacetilasas (HDAC) y alteraciones en la metilación del ADN. Estas propiedades del ácido valérico en la HDAC también tienen un amplio espectro de actividad anticancerígena, con una alta citotoxicidad para el cáncer de hígado en ensayos de proliferación celular, formación de colonias, cicatrización de heridas, invasión celular y formación de esferoides en 3D. Algunos modelos de ratón demostraron que la administración sistemática de ácido valérico encapsulado en nanopartículas a base de lípidos, disminuye significativamente la carga tumoral, mejorando la tasa de supervivencia [13].

Se ha descrito ampliamente el proceso de la apoptosis y su función biológica en la patogenia de varias enfermedades tales como el cáncer, trastornos metabólicos, neuropatías, lesiones miocárdicas y alteraciones del sistema inmunitario. Tomando como premisa que aproximadamente el 50% de los tumores humanos están asociados a mutaciones del gen p53, es de vital importancia el estudio de los mecanismos de apoptosis en el proceso de carcinogénesis, así como el efecto de principios activos en estos mecanismos, en pro de la identificación de posibles blancos terapéuticos. Las señales de apoptosis están reguladas por diferencias en la expresión de proteínas promotoras e inhibidoras, especialmente los miembros de la familia Bcl-2 que están integrados por: Bcl-2, Bax, Bad, Bcl-X1, Bcl-Xs, Mcl-1 y algunas proteínas efectoras como las caspasas. Es necesario conocer la expresión de proteínas y su incremento y disminución a expensas de

posibles tratamientos que puedan ser coadyuvantes en el proceso de muerte celular en células cancerígenas.

En virtud que existen diversos mecanismos por dilucidar y tomando en consideración que se han reportado otras especies de valeriana propias de la región, que pudiesen estudiarse en modelos celulares, el presente estudio planteó como objetivo determinar el potencial terapéutico *in vitro* de *Valeriana rígida* y *Valeriana decussata* en células MCF-7, mediante la determinación de la capacidad pro-apoptótica y citotóxica del extracto de sus hojas, tallos y raíces.

MATERIAL Y MÉTODOS

Preparación de extractos

Los especímenes de valerianas se recolectaron en el cerro Igualata, ubicado en el cantón Quero, perteneciente a la provincia de Tungurahua-Ecuador, previa autorización emitida por la Dirección Provincial del Ambiente Tungurahua N°06-2018-IC-FLO-FAU-DPAT-VS. A cada una de las especies se les evaluó las características organolépticas y físicas; como estado, olor y estructura de hojas, tallos, flores y raíces. El material vegetal recolectado fue transportado al laboratorio de la Facultad de Ciencias e Ingeniería en Alimentos y Biotecnología para llevar a cabo el proceso de lavado y secado a 60°C. Luego se pulverizaron, tamizaron y se almacenaron a temperatura ambiente hasta su uso posterior. Para la obtención del extracto, se resuspendió el material vegetal pulverizado en PBS 1X estéril relación 1:20, después se homogenizó con un vórtex. Posteriormente, cada extracto fue transferido a otro tubo estéril de 15 mL a través de un filtro de 0,22 μm, con el objetivo de eliminar contaminantes o material seco y así conseguir la esterilidad. Se obtuvo un extracto de tipo acuoso con PBS en concentración 1X en relación 1:20 con los extractos de Valeriana, los cuales presentaron una coloración marrón de poca viscosidad. Se escogió este tipo de extracto debido a la composición bioquímica de las valerianas que, a la habitual administración farmacológica y el PBS como solución tampón, mantiene la presión osmótica y el equilibrio de las células. Todos los extractos se mantuvieron en congelación (-20°C).

Cultivo Celular

Para el desarrollo de la investigación se empleó la línea celular MCF7 ATCC®HTB-22™ derivada del cáncer de mama humano. Las células se cultivaron en frascos Roux de 75mL, en medio DMEN (Dulbeccos Modified Eagle Medium), suplementado con 10% de suero fetal bovino (FBS), 1% de penicilina/estreptomicina (100 μg/mL) y 1% de glutamina. Las células fueron incubadas a 37°C, 5% de CO_2 y atmosfera húmeda. Los pases celulares se realizaron en base a la proliferación y población celular contenida en los frascos, con una confluencia entre el 80 y 100%. Para el contaje celular se utilizó una cámara de Neubauer siguiendo el protocolo establecido, a la cual se le añadieron 10 μL de la disolución final teñida con "azul tripán" y se leyó en un microscopio óptico (magnificación 10X). Finalmente, se obtuvo el número de células viables por cada mL de suspensión.

Análisis de viabilidad celular

Para calcular la citotoxicidad de los extractos en células MCF-7, se empleó el ensayo MTT (3- (4,5-dimetiltiazol-2-il) -2,5-difeniltetrazolio) de Invitrogen™ (USA). El tratamiento con los extractos se realizó a diferentes concentraciones en diluciones seriadas, en función de la base logarítmica 10 (Rango 10^{-8} a 10^{-1} de acuerdo con la concentración inicial de proteínas contenidas en cada extracto: *decussata* raíz (DDR) (4mg/mL), *decussata* hoja y tallo (DHT) (6,3 mg/mL), *rígida* hoja y tallo (RHT) (3,6 mg/mL) y *rígida* raíz (RR) (3,2 mg/mL). Seguidamente se agregaron los extractos en las células cultivadas en placas de 96 pocillos con una densidad de 1,0 x 10^4 cel/pozo y se leyeron en un lector de placas Perkin Elmer Víctor X3 (USA), según el protocolo descrito por

la casa comercial [17]. Todos los experimentos se realizaron por duplicado. El valor de la concentración inhibitoria media (IC50) se determinó utilizando el programa GraphPad Prism V7.0 (Software Inc., San Diego, CA, USA).

Determinación de Proteínas

Para la cuantificación de proteínas se empleó el método Bradford, se elaboró la curva de calibración con albúmina de suero bovino (BSA) a 0,25, 0,50, 0,75, 1,00, 1,25, 1,50 y 1,75 mg/mL. se realizó la lectura de las absorbancias a 595 nm en un espectrofotómetro de microplacas (PerkinElmer Víctor X3, USA).

Expresión Génica

Para comparar los datos experimentales, se realizó una RT-qPCR para determinar la expresión de Bax, Bcl-2 y p53 a partir del ARN obtenido de las células MCF-7 tratadas y del control (no tratadas).

El proceso se inició con la extracción de ARN de la suspensión celular, utilizando el estuche PureLink® RNA Mini Kit, según el protocolo del fabricante [18], se determinó el valor de concentración (ng/μL) y se almacenó a -80°C (12)**.** El ADNc se sintetizó a partir del ARN total utilizando el protocolo Maxima First Strand cDNA Synthesis (ThermoScientific) [19].

Finalmente, para realizar la RT-qPCR se utilizó el protocolo GoTaq® qPCRMaster. En una tira de tubos Eppendorf se agregaron 10 μL de Mix (2X), 1 μL de Forward Primer, 1 μL de Reverse Primer (Tabla 1), 1,6 μL del templado de ADNc por cada tubo (según la reacción) y se completó hasta 20 μL con agua libre de ARNasa. Los tubos se centrifugaron y fueron colocados en el Termal Cycler (CFX96** Real-time System, USA). El equipo se programó según el protocolo de Promega (Promega GoTaq®, 2014). Las condiciones de amplificación fueron: activación de la polimerasa a temperatura de 95°C durante 30 segundos, desnaturalización del ADNc a 95°C durante 15 segundos; amplificación a 58°C durante 25 segundos en un total de 32 ciclos.

Análisis estadístico

Los resultados obtenidos se analizaron con el programa GraphPadPrism V7.0 (Software Inc., San Diego, CA, USA). Se expresaron como media y desviación estándar ($\bar{x}\pm$ DS). Para el análisis de varianza se utilizó ANOVA de un solo factor con post test de Bonferroni, considerando significativos los valores de $p \leq 0,05$ con un 95% de límite de confianza.

RESULTADOS

Efecto de los extractos en la viabilidad de las células neoplásicas

La Fig. 1 muestra el efecto de los extractos de DR y DHT en la viabilidad de las células neoplásicas. Si bien se observaron diferentes grados de efecto inhibitorio según las dosis de los extractos, el mayor efecto inhibitorio se encontró en la dosis de 10^{-3} mg/mL. La Fig. 2 muestra el efecto inhibitorio de los extractos de la RR y la RHT. La RHT

Tabla 1
Secuencia de Primers.

Gen	Forward Primer	Reverse Primer
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGA
ACTINA	CGGTTCCGATGCCCTGAGGCTCTT	CGTCACACTTCATTGATGGAATTGA
BAX	CAAGACCAGGGTGGTTGGG	ATCTTTGTGGCGGGAGTG
Bcl-2	CATGTGTGTGGAGAGCGTCAA	GCCGGTTCAGGTACTCAGTCA
p53	GACGGTGACACGCTTCCCTGGATT	GGGAACAAGAAGTGGAGAATGTCA

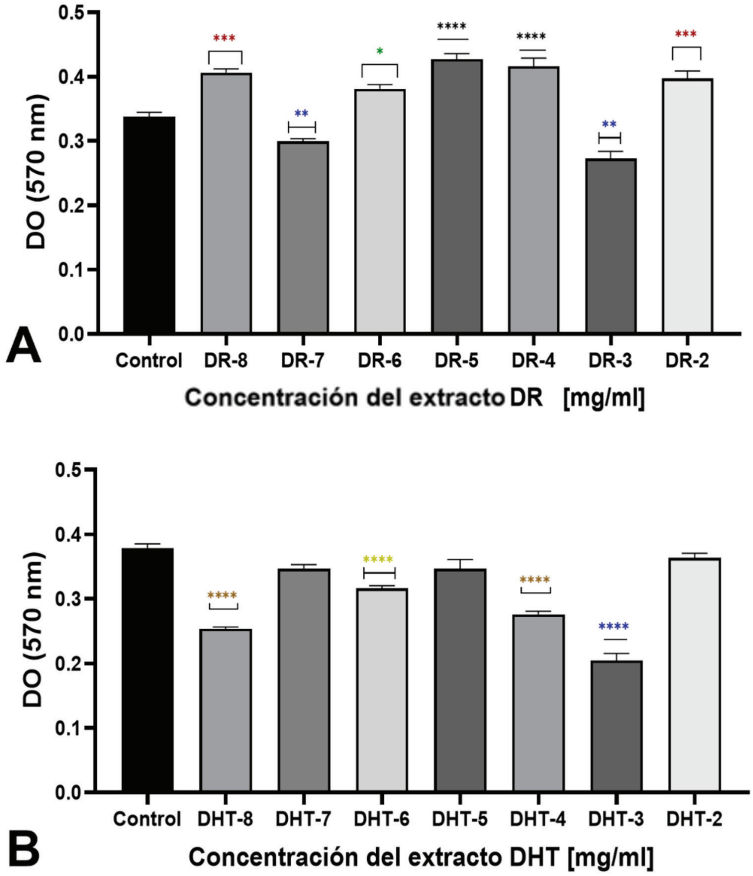

Fig. 1. Ensayo de viabilidad de los extractos de valeriana en células MCF-7. Las células se sembraron a $1x10^4$ células por pozo y se trataron con dosis crecientes (10^{-8} mg/mL a 10^{-1}mg/mL) de *Valeriana decussata* raiz (**DR:** 4mg/mL) (A) y *Valeriana decussata* hojas y tallos (**DHT:** 6,3 mg/mL) (B). Los experimentos se realizaron por triplicado para evaluar la concentración inhibitoria media máxima (IC50: 10^{-3} mg/mL) para los extractos de plantas. Tratamiento Vs. control (α= 0,05, *P=<0.05; **P=<0.01; ***P<0.001; ****P<0,0001).

presentó fluctuaciones en el efecto inhibitorio de acuerdo con las dosis (Fig. 2A), sin embargo, todas las dosis de RR fueron similarmente inhibitorias (Fig. 2B). Ambos extractos tuvieron el mayor efecto inhibitorio en la dosis de 10^{-3} mg/mL.

Expresión génica de proteínas vinculadas con la apoptosis

No se observó expresión génica de la proteína p53 en las células tratadas con los extractos, sin embargo y en menor proporción a las células no tratadas, se apreció expresión de Bax y Bcl-2 en las células neoplásicas tratadas, encontrándose mayor expresión de Bax (Figs. 3 A y B). Como es esperado índice Bax/Bcl-2 se observa elevado en los diferentes tratamientos con los extractos, presentándose los mayores valores en los tratamientos con extractos de *Valeriana decussata* (HT) y *Valeriana rígida* (RR) (Fig. 3C).

El gen de control endógeno GAPDH no presentó variación significativa en su expresión génica después de la aplicación de los tratamientos de *Valeriana decussata* y *Valeriana rígida* (Fig. 4). La expresión relativa normalizada de la proteína Bcl-2 y Bax se muestran en la (Fig. 4).

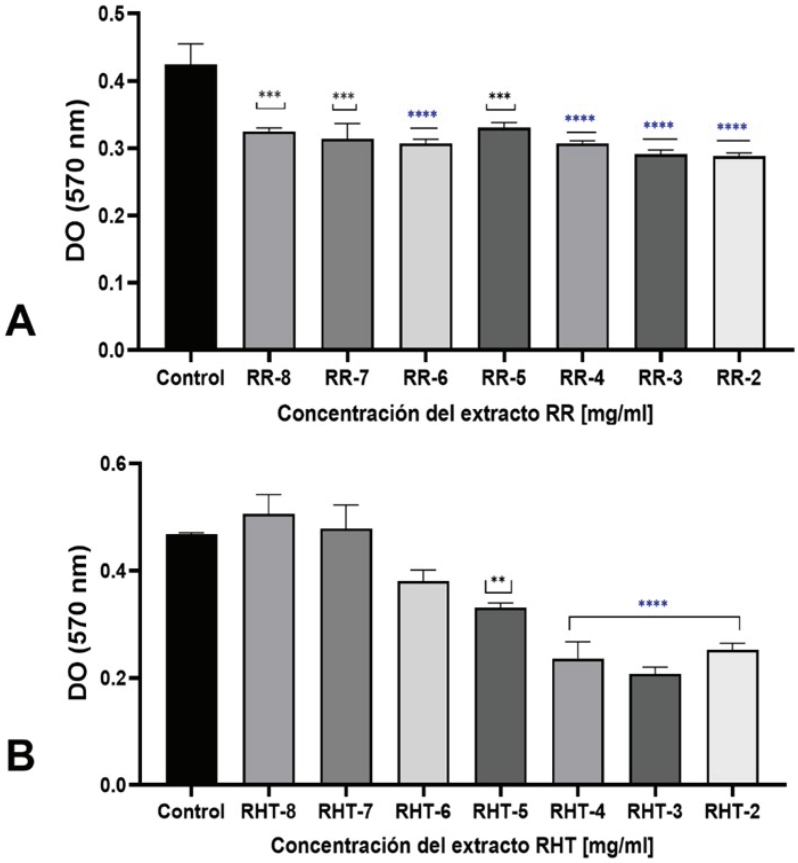

Fig. 2. Ensayo de viabilidad de los extractos de valeriana en células MCF-7. Las células se sembraron a $1x10^4$ celulas por pozo y se trataron con dosis crecientes (10^{-11} mg/mL a 10^{-1} mg/mL.) de Valeriana rígida raíz (RR: 3,2 mg/mL) (A) y Valeriana rígida hojas y tallos (RHT: 3,6 mg/mL) (B). Los experimentos se realizaron por triplicado para evaluar la concentración inhibitoria media máxima (IC50: 10-3 mg/mL) para los extractos de plantas. Tratamiento Vs. control (α= 0,05,**P<0,05; ****P<0,0001).

DISCUSIÓN

Es de gran importancia el estudio de compuestos fitoquímicos en pro de la búsqueda de alternativas o coadyuvantes en la terapéutica convencional del cáncer. A la fecha se han atribuido múltiples propiedades a las valerianas principalmente a la *V. officinalis*; sin embargo, son muchas las variedades que quedan por estudiar, algunas endémicas de la Sierra del Ecuador.

En la actualidad, no se conoce un efecto real de la Valeriana sobre células cancerígenas, ya que su uso terapéutico se ha limitado al sistema nervioso para el tratamiento del insomnio y la ansiedad. Sin embargo, la Valeriana tiene compuestos que potencian la interacción sinérgica de otros medicamentos depresores del SNC (sistema nervioso central) al inducir la eficacia del tratamiento [21]. También existen investigaciones de la inducción de la actividad ansiolítica, la relación con el efecto del ácido valórico y la interferencia del ácido acetoxi valerénico. Garrido en el 2007 determinó mediante la técnica de cromatografía de gases, que la *Valeriana Prionophylla* contiene ácido valerénico, acetoxivalerénico e hidroxivalerénico y los Valepotriatos contienen derivados como el baldrinal, homobaldrinal, acevaltrato, didrovaltrato, didrovaltrato, isovaltraro y valtrato. La cantidad de compuestos y ácidos

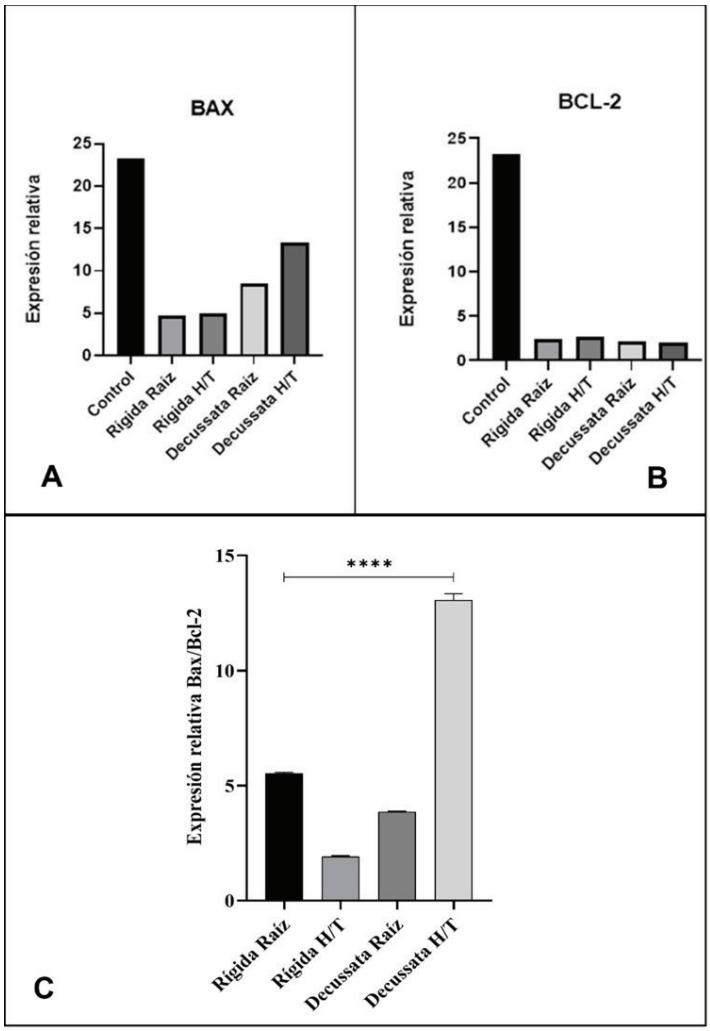

Fig. 3. Expresión relativa normalizada de las proteínas Bax (A) y Bcl-2 (B) según el método ΔΔCt: Se compara la expresión relativa del gen endógeno GAPDH vs la expresión génica de las células MCF-7. La expresión relativa Bax/Bcl-2 se muestra en el panel C (****P≤0,0005), (***P≤0,005), (**P≤0,05).

tuvo diferencias en función de los extractos a partir de hojas y raíces [22].

El ensayo de MTT evidenció un potencial efecto de los extractos en la proliferación celular y el bajo nivel de citotoxicidad, mostrando el comportamiento y funcionabilidad de las células MCF-7 sometidas a tratamientos [23].

Los extractos inhiben la proliferación celular de forma dependiente a una concentración determinada en la dilución logarítmica 10^{-3} [24]. Al comparar el comportamiento celular con cada extracto se evidenció que para ambas valerianas se obtuvieron mejores resultados de los extractos a partir de hojas y tallos. Esto puede ser ocasionado por composición bioquímica presente en las plantas que inducen a la inhibición en la proliferación celular.

La proliferación evidenciada en concentraciones logarítmicas diferentes a 10^{-3} posiblemente pudo ser ocasionada por la interacción entre las células y el extracto acuoso como lo mencionaron Flores y Martínez en 2019 [25] dependiendo del tipo de planta y el extracto acuoso que se utilice, la prolifera-

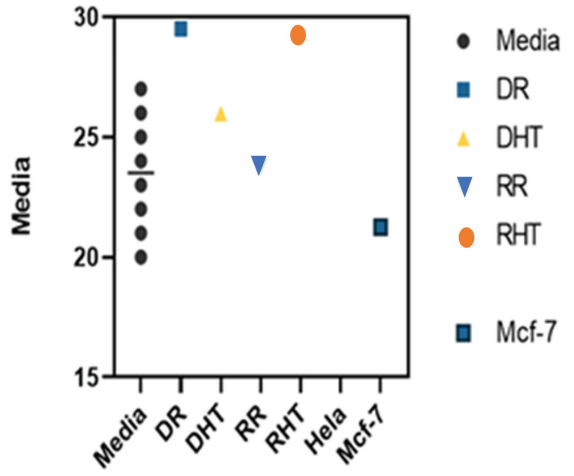

Fig 4. Análisis de los valores de Cts obtenidos para el gen GAPDH: Se analizó y validó al GAPDH como gen calibrador y de referencia. (P>0,05) Las medias de las muestras estuvieron en el margen de la media para GAPDH (P>0,05). *Valeriana decussata* raiz **(DR)** *Valeriana decussata* hojas y tallos **(DHT)** *Valeriana rigida* raiz **(RR)** *Valeriana rigida* hojas y tallos **(RHT).**

ción celular variará para diferentes concentraciones.

El análisis de varianza mostró diferencias significativas entre concentraciones, dependientes de las especies de valeriana y la estructura vegetal con la que se preparó el extracto y los ensayos; sin embargo, según otros estudios realizados con valeriana, las porciones subterráneas de la planta contienen mayor concentración de compuestos citotóxicos, lo cual podría explicar que en estas investigaciones la citotoxicidad fue mayor. Es importante mencionar que algunos extractos no presentaron diferencia significativa lo cual se pudo originar por la composición bioquímica de la planta, además que las especies incluidas no han sido estudiadas previamente. Está variación puede ser ocasionada como una respuesta de las células ante sustancias extrañas, que considera como peligrosas para su interior o por compuestos presentes en la valeriana que inducen la proliferación, por lo cual es relevante realizar a futuro ensayos fitoquímicos que muestren la composición los extractos.

Los resultados de la expresión génica mostraron variaciones de las proteínas que inducen la apoptosis; estos cambios pueden ser ocasionados por la composición bioquímica presente en las raíces, hojas y tallos de las Valerianas, de allí la importancia de haber realizado la presente investigación. Sin embargo, es necesario profundizar en la composición química y los grupos funcionales presentes en las diferentes especies de Valeriana que estarían incidiendo directamente en la capacidad pro-apoptótica de las células MCF-7.

Se pudo observar que la expresión de genes pro-apoptóticos Bax y Bcl-2 para RR y DHT no superaron el valor alcanzado por el control, esto es un indicativo de la ausencia de compuestos inductores de dichos genes o la represión de estos. Sin embargo, para las demás muestras, se evidenció un aumento de expresión con relación al control. Los valores bajos de expresión génica pueden ser debidos a varios factores, sin embargo, se cree que están principalmente relacionados con el solvente y el método usados para obtener el extracto. Según Páez-Hernández y col. [26], la mayor cantidad de compuestos con actividad quimiprotectora e inductora de los genes Bax y Bcl-2, se encuentra en los aceites esenciales de las plantas.

La relación de las proteínas Bcl-2 y Bax es muy importante en la activación de la apoptosis ya que esta depende de la regulación de ambas. Bcl-2 es una familia de proteínas que presenta actividad anti-apoptótica, que ha generado el estudio de su regulación en la apoptosis y la respuesta celular ante diferentes terapias contra el cáncer. Por su parte, Bcl-2 puede inducir o reprimir la liberación de factores indispensables para la apoptosis como citocromo c y el AIF (Factor inductor de apoptosis) [27]. La proteína pro-apoptótica Bax es una subfamilia homóloga de Bcl-2. Para que se desencadene el proceso de apoptosis es necesaria la regulación de Bax/Bcl-2. Bax tiene como función principal

la permeabilidad de la mitocondria. Estudios realizados por Hussein y Chavi en 2015, demostraron que la inducción de apoptosis en células MCF-7 era ocasionada por la regulación de Bax [28], lo cual está relacionado con la inducción apoptótica de p53 que regula a otras proteínas pro-apoptóticas como Bax. En este estudio se pudo apreciar incremento en la expresión génica de Bax en relación con la expresión de Bcl-2, lo que conduciría a la permeabilización de las mitocondrias con el subsiguiente proceso apoptótico [29].

La proteína p53 no se expresó en las células tratadas con extractos de valeriana, esto pudo ser ocasionado por la interacción de los compuestos presentes en las especies de valeriana que indujeron a la activación de proteínas celulares, que pudieron afectar el funcionamiento de p53. Esto puede ser un indicativo de la proliferación celular a ciertas concentraciones de los extractos, ya que, si no se activa la proteína por daño genético o alteraciones en el mecanismo de control, puede ocasionar la proliferación celular [30].

La proteína p53 cumple diversas funciones biológicas y es un factor indispensable en los procesos intra y extracelulares. En el presente estudio, se evaluó la capacidad de inducción al suicidio celular apoptótico. Diferentes factores de estrés inducen a que la proteína p53 cruce la mitocondria, active la expresión de genes pro-apoptóticos e inhiba la expresión de genes anti-apoptóticos [26]. Un estudio realizado en células MCF-7 con extracto de *Trifolium Pratens L.*, demostró que inducía apoptosis mediante la regulación de la proteína p53, de manera dependiente de la dosis y del tiempo del extracto, induciendo autofagia y apoptosis [31]. En nuestro estudio, la falta de expresión génica de la proteína p53 en las células tratadas con los extractos, sugiere un papel no relevante de esta proteína.

En función de los resultados obtenidos se puede inferir que las diferentes especies de Valeriana estudiadas, contienen compuestos que inducen la expresión de proteínas que dirigen la apoptosis en células cancerígenas de mama. Se requieren más estudios cuantitativos que permitan esclarecer el efecto de estos extractos en otros tipos de cáncer y la verificación cuantitativa del desarrollo de la apoptosis, debido a la expresión de las proteínas pro-apoptóticas como mecanismo de acción de estos sustratos. Este estudio establece la posibilidad del uso de los derivados de la valeriana para optimizar la terapia contra este tipo de cáncer.

AGRADECIMIENTO

A la Universidad Técnica de Ambato, a través de la Dirección de Investigación y Desarrollo (DIDE) por el financiamiento de los proyectos: "Estudio etnobotánico y bioensayos de plantas medicinales relacionadas con el tratamiento de cáncer de cérvix en la ciudad de Ambato, provincia de Tungurahua, Ecuador, aprobado con resolución: 0904-CU-P-201, y "Caracterización morfológica molecular fitoquímica y métodos de propagación en especies silvestres del género valeriana de la provincia de Tungurahua" aprobado con la resolución: 1569-CU-P-2017.

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Conflictos de interés

Los autores declaran no tener ningún conflicto de interés.

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Invest Clin 63(4): 400 - 413, 2022 https://doi.org/10.54817/IC.v63n4a07

Fibrolipomatous hamartoma of the median nerve: a case report and literature review.

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Keywords: fibrolipomatous hamartoma; the median nerve; the index finger; surgery; oncology.

Abstract. Fibrolipomatous hamartoma (FLH) of the nerve, also known as lipomatosis of the nerve, neurofibrillary lipomatous lesion, or intraneural lipoma, is a rare benign soft tissue tumor which mainly occurs in the nerves of the upper limb, especially in the median nerve. In April 2021, a 30-year-old male patient was secondly admitted to our hospital and underwent his third surgery, due to the recurrence of a mass and pain in the right palm, noticeable swelling and numbness of the right index and ring fingers, and limited flexion and extension activities of the right ring finger. He first visited our hospital in December 2017 due to a mass and pain in the right palm and swelling and numbness of the right index and ring fingers. When the clinician asked for the patient medical history, his parents stated that his right middle finger was swollen after birth. When the patient was ten years old; he was diagnosed with "macrodactyly" at the local county hospital, not in our hospital, and subsequently, the middle finger was amputated at the metacarpophalangeal joint level at the local county hospital. The postoperative pathological examination was not performed at that time, which was the first surgery the patient received. FLH is clinically rare, and its exact epidemiology and etiology are poorly understood. FLH is highly suspected in cases where a painless mass is present in the wrist, combined with macrodactyly. Magnetic resonance imaging and pathological examination are helpful in clarifying the diagnosis. Although FLH is a benign tumor, an individual treatment plan is the best choice according to the severity of the patient's symptoms. Therefore, further exploration and understanding of this disease by clinicians radiologists, and pathologists is necessary.

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Hamartoma fibrolipomatoso del nervio mediano: reporte de un caso y revisión de la literatura.

Invest Clin 2022; 63 (4): 400 – 413

Palabras clave: hamartoma fibrolipomatoso; nervio mediano; dedo índice; cirugía; oncología.

Resumen. El hamartoma fibrolipomatoso (FLH) del nervio, también conocido como lipomatosis del nervio, lesión neurofibrilar lipomatosa, o lipointraneural, es un tumor benigno de tejido blando poco frecuente, que se presenta principalmente en los nervios del miembro superior, especialmente en el nervio mediano. En abril de 2021, un paciente masculino de 30 años fue ingresado por segunda vez en nuestro hospital y sometido a su tercera cirugía debido a la recurrencia de una masa y dolor en la palma derecha, evidente hinchazón y entumecimiento de los dedos índice y anular derecho y limitadas actividades de flexión y extensión del dedo anular derecho. En diciembre de 2017, visitó por primera vez nuestro hospital debido a una masa y dolor en la palma derecha, y a la hinchazón y entumecimiento de los dedos índice y anular derecho. Cuando el clínico preguntó la historia clínica del paciente, sus padres declararon que su dedo medio derecho estaba hinchado después del nacimiento, y cuando el paciente tenía 10 años, fue diagnosticado con "macrodactilia" en el hospital local del condado, no en nuestro hospital Posteriormente, el dedo medio fue amputado a nivel de la articulación metacarpofalángica en el hospital comarcal local, pero no se realizó la patología postoperatoria en ese momento, siendo ésta la primera cirugía a la cual se sometió el paciente. La FLH es clínicamente rara, y su epidemiología y etiología exactas no se entienden bien. En los casos que presentan una masa indolora en la muñeca, combinada con macrodactilia, se sospecha de FLH. La resonancia magnética y la patología son útiles para aclarar el diagnóstico. Aunque la FLH es un tumor benigno, el plan de tratamiento individual es la mejor opción de acuerdo con la gravedad de los síntomas del paciente. Por lo tanto, es necesaria una mayor exploración y comprensión de esta enfermedad por parte de médicos, radiólogos y patólogos.

Received: 24-04-2022 Accepted: 10-05-2022

INTRODUCTION

Fibrolipomatous hamartoma of the nerve (FLH), also known as lipomatosis of the nerve, neurofibrillary lipomatous lesion, or intraneural lipoma, is a rare benign soft tissue tumor, which mainly occurs in the nerves of the upper extremity, especially in the median nerve and its branches [1-3]. In addition, a few occur in the ulnar nerve, radial nerve or sural nerve, and a few are accompanied by macrodactyly [3]. Clinically, it should be avoided to be confused with simple lipoma and neurofibromas. Magnetic resonance imaging (MRI) remains the gold standard for the diagnosis of FLH. The pathological morphology of FLH is characterized by the proliferation of fibrofatty tissues around the nerve, infil-

tration of the epineurium and perineurium, and obvious thickening and deformation of the affected nerve. As we know, there are few reports on fibrolipomatous hamartoma of the peripheral nerve in domestic and international literature. In this paper, we report a rare case of fibrolipomatous hamartoma of the median nerve and performed a literature review on this condition.

Case presentation

This study was conducted in accordance with the declaration of Helsinki and approved by the Ethics Committee of Honghui Hospital, Xi'an Jiaotong University. Written informed consent to publish the clinical details and images of the patient was obtained.

In April 2021, a 30-year-old male patient was secondly admitted to our hospital due to the recurrence of a mass and pain in the right palm, obvious swelling and numbness of the right index finger and ring finger, and limited flexion and extension activities of the right ring finger.

In December 2017, he came to our hospital for the first time because of a mass and pain in the right palm, and swelling and numbness of the right index figure and ring finger. When the clinician asked about the patient's medical history, his parents stated that his right middle finger was swollen after birth, and when the patient was 10 years old, he was diagnosed with "macrodactyly" in the local county hospital, not in our hospital. Subsequently, the middle finger was amputated at the metacarpophalangeal joint level in the local county hospital, which was the first surgery the patient received. The postoperative pathological examination was not performed at that time. None of the family members had this kind of disease.

In December 2017, he underwent his second surgery in our hospital. The preoperative physical examination in our hospital showed that the right middle finger was absent. The whole right index finger was significantly swollen and had a wide nail. The right ring finger, proximal to the right palm was swollen, there was a soft tissue mass of 10cm*3–5cm on the radial side of the right palm and the palmar side of the right wrist and a mass of 2cm*1cm on the ulnar side of the distal segment of the right thumb (Figs. 1A and 1B), The flexion and extension

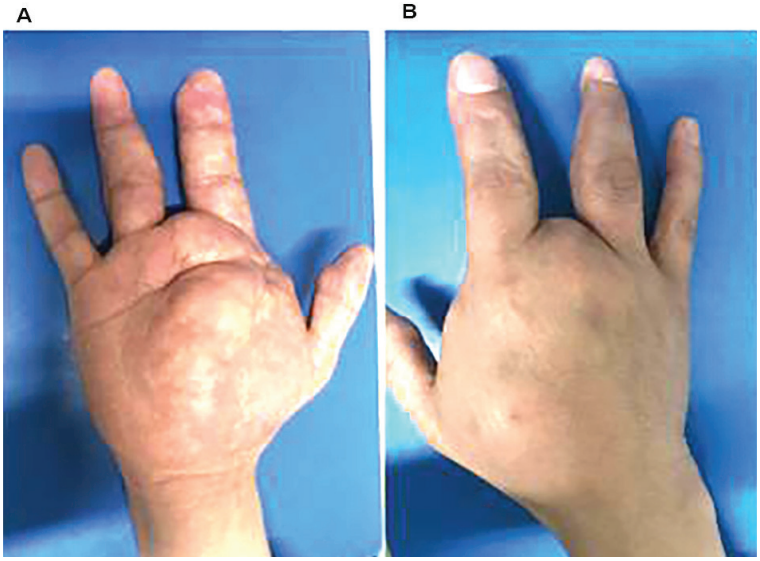

Fig. 1. Preoperative volar side of the right hand in A and dorsal side of the right hand in B showing the absent right middle finger, a soft tissue mass on the right palm, significantly swollen right index finger body, and partially swollen right ring finger.

activities of the right index figure and ring finger were limited, accompanied by skin numbness, the right hand was restricted in making a fist, and the peripheral blood flow was normal. The auxiliary examinations, including B-scan ultrasonography, X films and computerized tomography (CT), were performed. The B-scan ultrasonography revealed intense hyperechogenicity in the soft tissue of the volar aspect of the right hand (Fig. 2). The X films revealed the absent right middle finger, the deformation of the third metacarpus head with decreased bone density, and the deformation of the articular surface of the middle phalanx head in the index finger (Figs. 3A and 3B). The CT revealed a solid lesion in the soft tissue of the right palm (Fig. 4).

Brachial plexus nerve blocking anesthesia was used. An S-shaped incision was made on the volar side of the right forearm and the right palm. Intraoperatively, a great amount of granular hyperplastic adipose tissue was observed (Figs. 5A, 5B, 5C, and 5D). The median nerve was dissected from the proximal to distal, and we found the enlargement of nerve bundles, the adhesion of the thickening epineurium, and the compression of transverse carpal ligament. We removed most of the hyperplastic fatty lumps, trimmed the epineurium, and performed open carpal tunnel release. After the open carpal tunnel release, the continuity of the median nerve was intact. A Z-shaped incision was made on the ulnar side of the right index finger and the thumb, and the radial side of the ring finger. The intrinsic nerves on the ulnar side of the index finger, and the radial side of the ring finger, had severe fatty infiltration distal to the proximal interphalangeal joint. We excised most of tumors under the microscope, and retained some of the nerve bundle branches and dorsal branches of the index and ring fingers. The postoperative pathological diagnosis was tumor-like hyperplasia of adipose tissue and degeneration and hyperplasia of the fibroblastic tissue of nerves (Fig. 6).

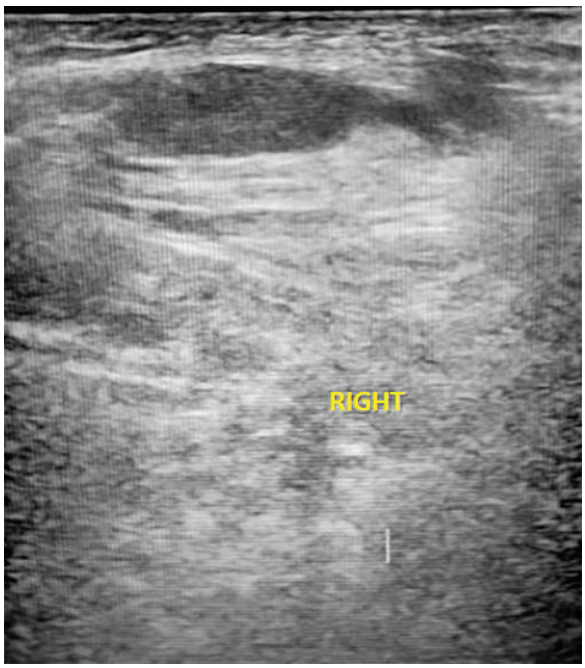

Fig. 2. Preoperative ultrasound showing dense echo in the soft tissue of the right palm.

After his second surgery, the patient was hospitalized again in April 2021, due to the recurrence of a mass and pain in the right palm, obvious swelling and numbness of the right index and ring fingers, and limited flexion and extension activities of the right ring finger. Physical examination upon admission suggested that the right middle finger was absent, and the incision of the right palm, the thumb, the index finger, and the ring finger healed well. There was an 8cm x 4cm irregular soft tissue mass on the radial side of the right palm and around the thenar eminence. The right index finger body was obviously swollen, with the deformation of the puffy distal phalanx. Scar contracture appeared on the ulnar side of the right ring finger, and some soft tissue thickening (Fig. 7). Flexion and extension activities were restricted in the right index finger, accompanied by skin numbness. In addition, the peripheral blood flow was normal. Auxiliary examinations, including B-scan ultrasonography, X films and magnetic resonance imaging (MRI), were performed. B-scan ultrasonography revealed thickening of local soft tissues of the right index finger,

Fig. 3. Preoperative X films of frontal position of the right hand (A) and lateral position of the right hand (B) showing the thickened soft tissue, the absent right middle finger, deformation of the third metacarpal head, and deformation of the articular surface of the middle phalanx of the index finger.

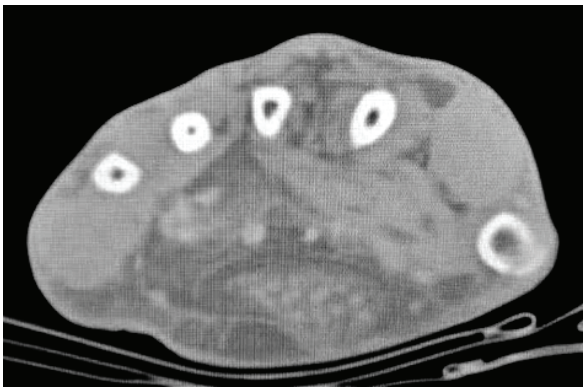

Fig. 4. Preoperative CT axial image of the right hand showing a solid mass in the soft tissue area of the right palm with hypodensity.

right ring finger, and right palm, and multiple strong echogenic and hypoechoic areas (Fig. 8). X films showed the absent right middle finger, enlargement and deformation of the third metacarpus head with reduced bone density, and osteophyte deformation of the proximal phalanx and middle phalanx of the index finger and the proximal phalanx of the ring finger (Figs. 9A and 9B). MRI showed abnormal signal shadows on the volar side of the right wrist, which suggested tumor recurrence, fat accumulation around the lesion and at the distal of the third metacarpophalangeal joint of the right hand, hypertrophy of the right index figure and ring finger, and the absence of the middle finger (Fig. 10A, 10B, and 10C).

The same S-shaped incision was made on the right palm and the volar side of the right forearm. Intraoperatively, an obvious adipose tissue-occupying lesion was observed, with uneven median nerve fiber bundles, severe fibrosis, and severe fatty and fibrous tissue infiltration (Fig. 11A, 11B, 11C, and 11D). We performed the dissection form the middle of the forearm to the fingers of the right hand to expose the normal range of the median nerve and the recurrent branch of the median nerve, and then we cut the intrinsic nerve at the proximal on the radial and ulnar sides of the thumb. Subse-

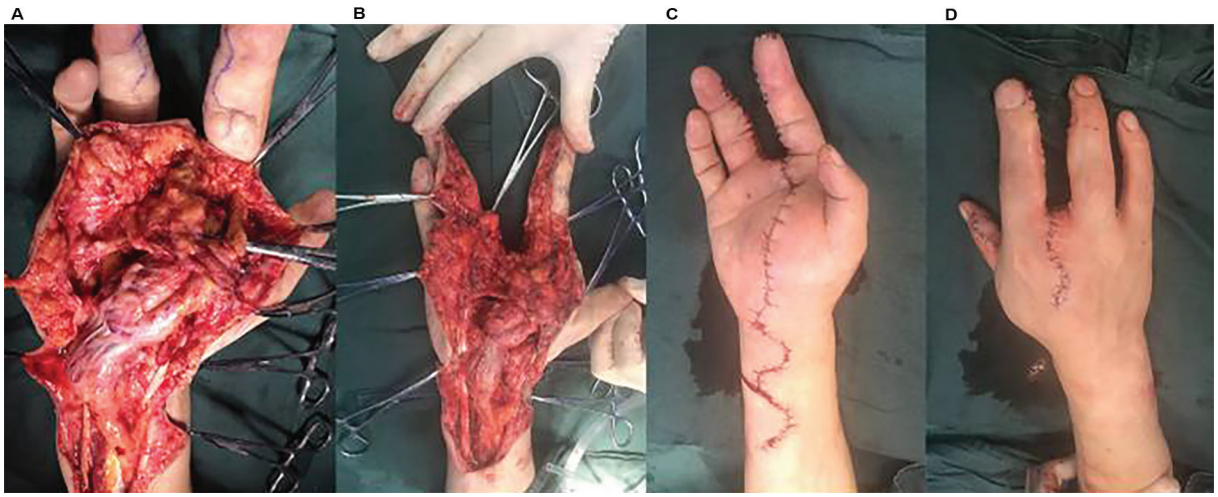

Fig. 5. Intraoperatively, the tumor extensively infiltrated the median nerve of the right wrist, and the digital nerves of the right index and ring finger (A and B); Postoperatively, the incision was sutured and the peripheral blood flow was good after loosening the tourniquet (C and D).

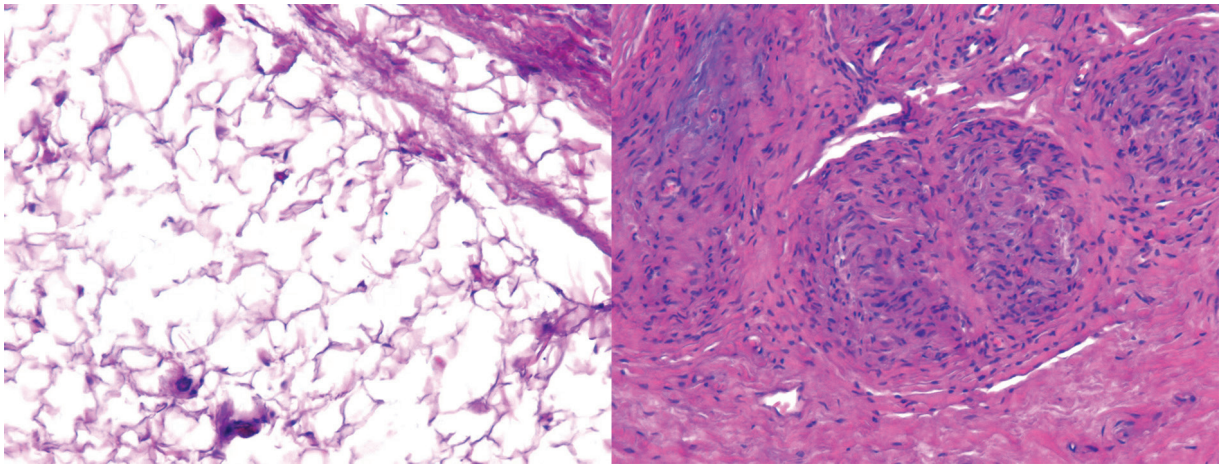

Fig. 6. Postoperative pathology showing tumor-like proliferation of fibrous and adipose tissue and partial degeneration of neural tissue.

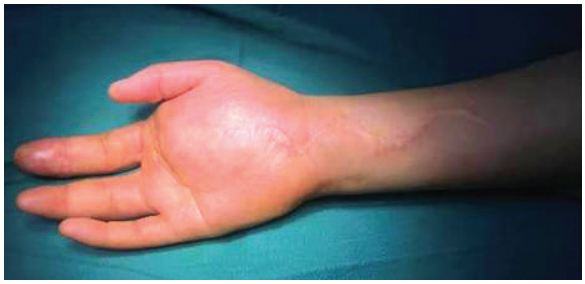

Fig. 7. Preoperative soft tissue tumor of the right palm.

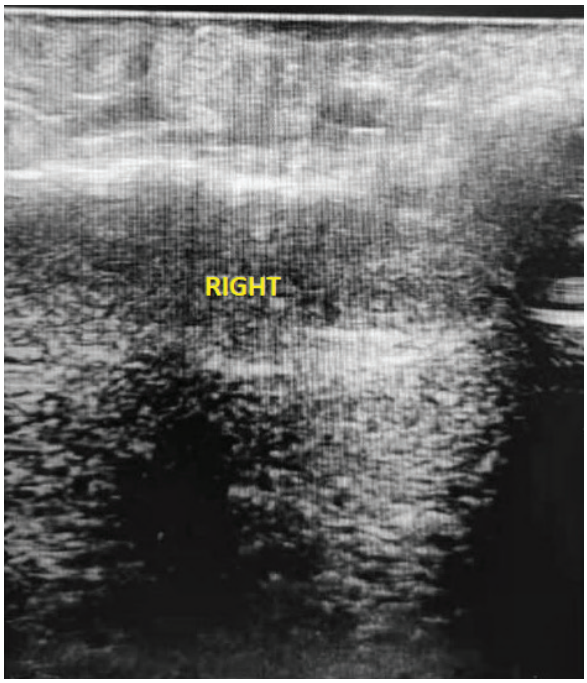

Fig. 8. Preoperative ultrasound showing local soft tissue thickening of the right palm with multiple hyperechoic and hypoechoic areas.

quently, the diseased median nerve and fat mass were completely removed. According to the length of the defective median nerve, the sural nerve of 36 cm was excised from the left calf, and folded into double strands to do the nerve transplantation and repair the defective median nerve. The superficial palmar arch artery had serious adhesion to the tumor tissue, and it ruptured during the separation, and was repaired via microscopic anastomosis. A Z-shape incision was made on the volar side of the right index finger and the radial side of the right ring finger, and then the adipose tissues were removed again for volume reduction. The proper digital artery on the radial side of the index finger at the proximal ruptured, and was repaired via microscopic anastomosis using ramus palmaris superficialis arteriae radialis. Postoperatively, the peripheral blood flow was good. The postoperative pathological diagnosis was tumor-like hyperplasia of fibrous fat and neural tissue (Fig. 12).

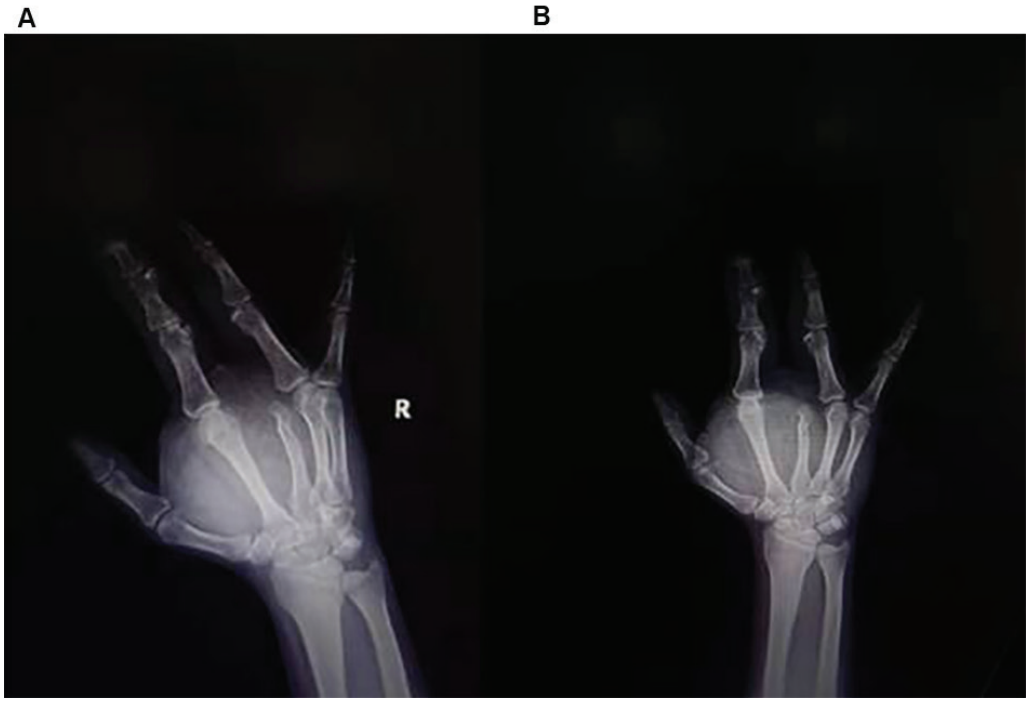

Fig. 9. Preoperative X films of oblique position (A) and frontal position (B) of the right hand showing soft tissue mass thickening of the right palm, and osteophyte deformation of the proximal and middle phalanges of the right index finger and the proximal phalanx of the ring finger.

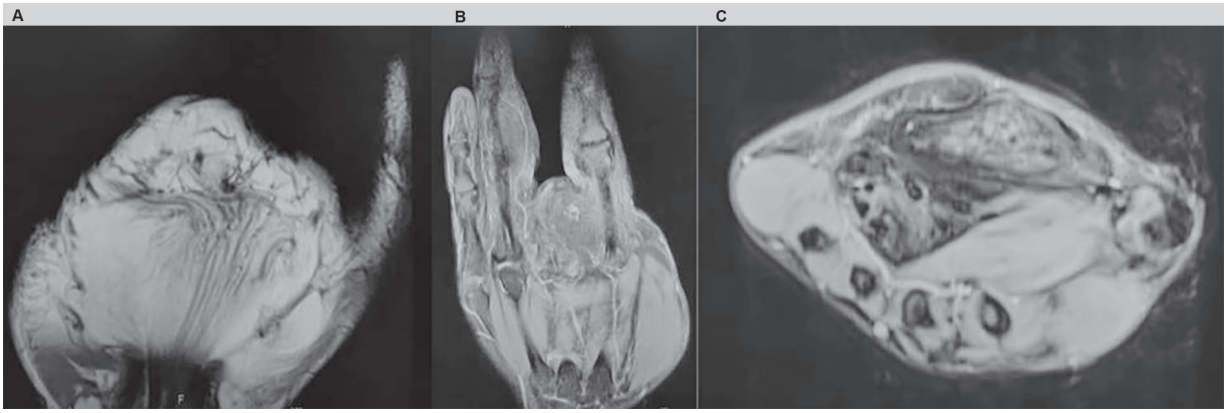

Fig. 10. For preoperative MRI, coronal T1 showing serpiginous structures-like changes (A), coronal T2 showing abnormal signal of the ring finger mass occupancy (B), and axial T2 showing coaxial cable-like appearance (C).

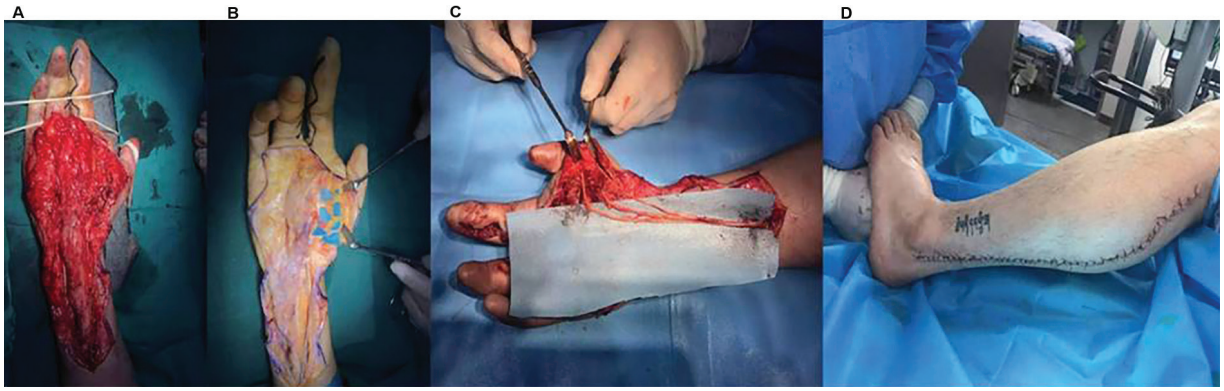

Fig. 11. Intraoperatively, the fatty tissue occupation was obvious, and the thickness of median nerve fiber bundles was uneven, with severe fibrosis as well as fatty and fibrous tissue infiltration (A and B); the median nerve was excised for sural nerve transplantation and repairment (C and D).

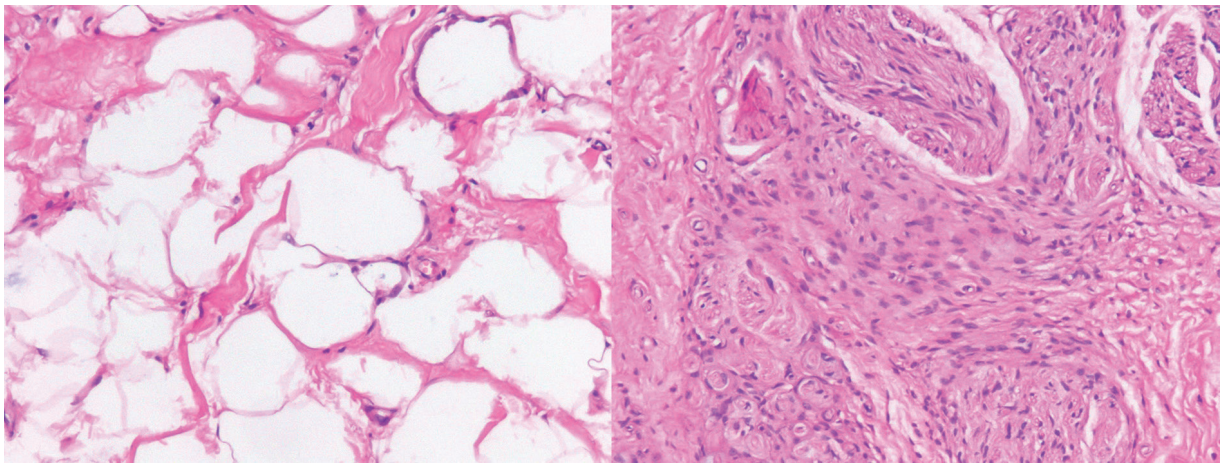

Fig. 12. Postoperative pathology showing tumor-like hyperplasia of fibroadipose tissue and fibrotic degeneration of neural tissue.

DISCUSSION

Definition and naming

FLH is a rare benign tumor, which mainly occurs in the nerves of the upper limbs. It originates from the abnormal growth of the fibroadipose tissue of the nerve sheath, resulting in the fusiform enlargement of the affected nerves [4,5]. The fibroid degeneration of the compressed nerve bundles is caused by the proliferation of adipose tissues [6]. The median nerve and its branches are involved in 85% of cases. FLH has several synonyms, such as lipofibromatous hamartoma, lipofibroma, fibrofatty proliferation of the nerve, fatty infiltration of the nerve, fibrolipoma of the nerve, intraneural lipofibroma, neural fibrolipoma, and macrodactylia fibrolipomatosis [5,7]. In the classification of soft tissue tumors by the World Health Organization (WHO) in 2013, fibrolipomatous hamartoma of the nerve, fatty infiltration, and neural fibrolipoma are all classified as lipomatosis of the nerve [1], which is different from intraneural lipoma in the clinical manifestations and treatment [8]. Due to the confusion of naming and the rarity of this disease, its diagnosis and treatment are complicated. The proper terminology for this lesion has been inconsistent in the literature and it is currently recommended that the most accurate term for this lesion is "fibrolipoma" [9], which best reflects the hamartomatous nature of this lesion. We also prefer the term "fibrolipomatous hamartoma of the nerve".

Epidemiology

This lesion was firstly reported by Mason in 1953 [2]. In 1969, Johnson and Bonfigilio firstly called this lesion as lipofibromatous hamartoma [10]. In 1994, Guthikonda *et al.* proposed a new classification for neural lipomatous tumours: (a) soft lipoma, (b) intraneural lipoma, (c) lipofibromatous hamartoma, and (d) macrodystrophia lipomatosa [11]. FLH commonly occurred in those under 30 years old [12]. This lesion in infants, children, and young adults was manifested by either no symptoms or compressive neuropathy [4]. In many cases, the mass has existed for many years before the onset of symptoms [13]. The incidence of the FLH with macrodactyly in women is twice as much as in men. The incidence of the FLH without macrodactyly in women is the same than in men, and the incidence of the FLH in the upper limbs is higher than that of the lower limbs [14-16]. In our reported case, the FLH occurred in a young man, involving unilateral median nerve and its branches, accompanied by macrodactyly.

Etiology and Pathogenesis

Although some potential etiologies have been described, the cause of this abnormal growth of the nerve is still unknown, which is possibly due to the proliferation and hypertrophy of fibroblasts and mature adipocytes in the epineurium [17]. A considerable number of cases occur at birth or in childhood. Many scholars believe that it may be a congenital disease caused by the abnormal development of the flexor retinaculum or transverse carpal ligament, especially in the carpal tunnel area or with macrodactyly. There are also cases with an overgrowth of bone tissue and skin soft tissue [12,18]. Some scholars believe that FLH is acquired and caused by reactions such as trauma or long-term chronic irritation [4,9]. The true etiology might be the combination of congenital factors and acquired and reactive factors, which stimulate the formation of the tumor [19]. Our reported case who had no history of trauma, presented with macrodactyly at birth, and his tumor recurred and continued to proliferate after two surgeries.

Although FLH is hyperproliferative, its behavior resembles that of a benign tumor, with no evidence of malignancy in confirmed cases and no familial cases. The FLH subtype has been reported to be associated with mutations in the PIK3CA pathway [20], similar to other hyperproliferative disorders. Neuroregional macrodactyly is a common comorbidity, and 20–66% of FLH cases experienced

this macrodactyly [14,15,21]. The exact relationship between the two remains elusive.

Clinical Manifestations

Typical FLH cases mainly occur in the first three decades of life, mostly at birth or in early childhood. Locally, gradually enlarging painless masses are the main symptom. 90% of cases are unilateral [12,22]. The course of the disease is variable, and the affected finger is symmetrically or asymmetrically thickened, which is often accompanied by dyskinesia. This kind of tumor mainly occurs in the upper limb, and the median nerve and its branches are the most commonly affected nerves, especially the median nerves at the distal end of the forearm and on the volar side of the wrist [12]. Pain, numbness, sensory abnormalities, and nerve entrapment syndrome usually occur in the late stage of this disease [23]. Approximately one-third of cases are accompanied with macrodactyly, which is commonly seen in the phalanges. However, there was a case report describing an elderly patient with macrodactyly in the metacarpal bone [12]. The case we reported presented with macrodactyly of the middle finger at birth. Unfortunately, because the clinicians in the local county hospital at that time had a lack of understanding of this disease, they performed the middle finger amputation to relieve pain and numbness of the patient.

Imaging Examinations

(1) B-scan ultrasonography suggests a dense-echo mass in the hypoechoic area. Nerve bundles are in the hypoechoic area, and adipose tissues are in the hyperechoic area. Color Doppler shows no blood flow signal [18].

(2) X-ray films show thickened soft tissues, and examine the bone for hypertrophy, hyperplasia, and other deformities [15].

(3) MRI is the gold standard for the diagnosis of this disease [24, 25], which is helpful for the differentiation and preoperative evaluation. Fat with high-signal intensity is showed on T1- and T2-weighted images. In the T1-weighted axial images, there is typical fat with high-signal intensity and nerves with a low-signal intensity and co-axial cable-like appearance. Coronal images showed serpiginous structures, and thickened nerve bundles were wrapped by adipose tissues, separated from each other, and evenly distributed in the nerve sheath. Fat in the T1-weighted images showed high-signal intensity, while fat in the T2-weighted images with fat suppression showed low-signal intensity. Fat was asymmetrically distributed, and sagittal images showed a spaghetti-like appearance. These specific features of MRI can be used as diagnostic criteria, even without a pathological biopsy [3,14]. For the case we reported, the preoperative MRI showed typical coaxial cable-like appearance and spaghetti-like serpiginous structures, which was very helpful for early diagnosis.

(4) Electromyography and nerve conduction examination are helpful to diagnose nerve compression lesions [12].

Pathological Examination

(1) General observation shows this kind of tumor is irregularly fusiform, its color is yellow or yellowish-brown, and the affected nerves may be elongated, thickened and uneven in thickness, which is caused by the excessive proliferation of mature adipocytes and fibrous tissue [19,25].

(2) Histological morphology shows excessive proliferation of the adventitial fibroadipose tissues of the affected nerves and extensive infiltration of the nerve bundles, and the adventitial fibroadipose tissues wraps, separates, and compresses these nerve bundles. In cases with a long course of disease, degenerative and atrophic changes of nerve tissues may occur. Fibrous tissues and perineurial cells located around the nerve bundle are arranged in concentric circles.[12,26] Unlike lipomas, the FLH presents infiltrative growth and fat is asymmetrically distributed between nerve bundles [12,26]. In the proliferative fibrous fat

tissue, the hyperplasia of medium and small blood vessels and lymphatics occurs focally, and hemangioma or lymphangioma-like changes may appear in some areas. In addition to the above-mentioned typical morphology, bone metaplasia may also occur in a few cases. Immunohistochemical markers have no special significance for the diagnosis of this disease, with being positive for CD34, S100, and vimentin and negative for epithelial membrane antigens, desmin, and glial fibrillary acidic protein antibodies [26].

Differential diagnosis

Clinically, many lesions need to be differentiated from FLH, such as neurofibroma, neurilemoma, neuroma, ganglion, lipoma, vascular malformation, intraneural neuroectodermal tumor, intraneural lipoma, and traumatic neuroma [6,18].

Treatment

Although FLH is a benign tumor, due to the rarity of this disease and the lack of randomized controlled studies, it is difficult to determine the best treatment for it. So far, the surgical approach of FLH has been controversial.

(1) Conservative treatment is an option for asymptomatic individuals [16,27], for whom there are no neurological symptoms such as pain, numbness or sensory abnormalities, and if the tumor is small enough not to affect the function and aesthetic of the affected limb. There is no evidence of malignancy. It primarily requires observation or a pathological biopsy [8].

(2) Carpal tunnel release is recommended to those with symptoms of median nerve compression, including traditional incision release, microscopic or arthroscopic carpal tunnel release, and epineurium incision and intraneural release as appropriate [4]. Nerve decompression can reduce pain, numbness and the risk of the sequelae of sensorimotor disorders [12]. It can also shrink the mass and improve thumb-to-palm strength [16,27]. If there are no characteristic imaging findings, a surgical exploration and an excisional biopsy are required to clarify the diagnosis.

(3) Microscopic resection is recommended if the tumor is large, and vascular injury and fibrosis further aggravate the nerve damage [13,16]. For cases of continuous and progressive deterioration of neurological symptoms, in order to maximize the preservation of nerve function, when the tumor resection was performed, interfascicular tumor dissection was performed together under a microscope. However, the dissection may cause segmental nerve ischemia [12,16,28]. Therefore, the specific indications for this technique are still controversial.

(4) Tumor and volume reduction, especially for cases combined with macrodactyly or swollen/deformed fingers, is recommended. In order to improve the appearance of the affected finger while preserving nerve function, tumor reduction surgery can be selected for soft tissue reduction, and if necessary, epiphyseal block, osteotomy correction, or the removal of the affected finger nerve was performed [26,28]. For some elderly patients, finger or limb amputation can be considered [26].

(5) The radical resection of the tumor is only performed in the cases of neurodisabling damage. Some scholars believe that this may lead to intolerable sensorimotor defects [7,12,28]. If the tumor involves the brachial plexus nerve, it is difficult to completely remove the tumor [12]. Postoperative fibrosis caused by fiber healing can significantly interfere with the nerve conduction [15,28], so that motor and sensory loss in the innervated area may occur. The improvement of motor functions requires tendonoplasty repair, and although nerve grafting is an option for sensory restoration, there is still no satisfactory solution.

For this case we report, when the patient firstly came to our hospital for surgery, a large number of proliferating fibers and fatty tumor tissues surrounding the nerve bundles were observed during operation. It was very challenging to completely

remove the tumor. Therefore, we decided to remove most of the tumor tissue to reduce its size, and loosen the carpal tunnel, while the median nerve and the finger nerves were preserved, which significantly reduced the patient's symptoms and improved the appearance of the affected finger. Three years after this operation, the tumor recurred and the patient was admitted again to our hospital for surgery. Due to extensive adhesion of the tumor tissue and the median neurofibrillary degeneration, the tumor and the median nerve of the diseased segment were removed, and then the sural nerve was cut to do the nerve grafting and repair. After this operation, the symptoms of the patient were relieved, and the feelings and activities of the palms and fingers were restored to the greatest extent.

CONCLUSION

The FLH is clinically rare, and its exact epidemiology and etiology are not well understood. In cases of a painless mass in the wrist, combined with macrodactyly, FLH is highly suspected. Magnetic resonance imaging and pathological examination are helpful to clarify the diagnosis. Although FLH is a benign tumor, the individual treatment plan is the best choice according to the severity of the patient's symptoms. Therefore, clinicians, radiologists, and pathologists need to further explore and understand this disease.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Ethics approval and consent to participate

This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethic committee of Honghui Hospital, Xi'an Jiaotong University.

Consent for publication

Written informed consent to publish the clinical details and images of the patient was obtained.

Conflict of interests

All authors declare they have no conflict of interests.

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Contributions Authors'

Y-T D, FX, Substantial contributions to the conception and design of the work.

YT D, QW, Y-P Z, D-KW, X-H O, FX, the acquisition, analysis, and interpretation of data for the work.

Y-T D, drafting the work.

Y-T D, QW, Y-P Z, D-K W, X-H O, F X, revising it critically for important intellectual content.

Y-T D, QW, Y-P Z, D-K W, X-H O, F X, final approval of the version to be published and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Invest Clin 63(4): 414 - 434, 2022 https://doi.org/10.54817/IC.v63n4a08

β-defensinas como posibles indicadores de la actividad inflamatoria en la enfermedad periodontal.

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Palabras clave: β-defensinas; epitelio bucal; enfermedad periodontal; gingivitis; periodontitis; gingivitis experimental.

Resumen. La enfermedad periodontal (gingivitis y periodontitis) es un proceso inflamatorio ocasionado por la actividad de bacterias patógenas y sus productos sobre el surco gingival, con la consecuente activación de la respuesta inmunitaria. La saliva y el fluido crevicular contienen una gran variedad de enzimas y factores antimicrobianos que están en contacto con la región supragingival y subgingival; entre ellos, las β-defensinas (hBDs). Las hBDs son péptidos catiónicos no glicosilados ricos en cisteína, producidos por las células epiteliales; tienen efecto antimicrobiano e inmunorregulador; de esta forma, contribuyen al mantenimiento de la homeostasis en los tejidos periodontales. Los cambios en la microbiota y en la respuesta inmunitaria de un periodonto sano a gingivitis y, finalmente, a periodontitis, es compleja. Su severidad depende de un equilibrio dinámico entre las bacterias asociadas a la placa, factores genéticos y ambientales. Los avances recientes han permitido comprender la implicación de las hBDs en la detección, el diagnóstico y la terapéutica de la enfermedad periodontal, así como la relación que hay entre la periodontitis y otras enfermedades inflamatorias. El objetivo de esta revisión es describir el efecto de las hBDs en la respuesta inmunitaria y su utilización como marcadores de la actividad inflamatoria de la enfermedad periodontal.

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β-defensins as possible indicators of inflammatory activity in periodontal disease.

Invest Clin 2022; 63 (4): 414 – 434

Keywords: β-defensins; buccal epithelium; periodontal disease; gingivitis; periodontitis; experimental gingivitis.

Abstract. Periodontal disease (gingivitis and periodontitis) is an inflammatory process caused by the activity of pathogenic bacteria and their products on the gingival sulcus, with the consequent activation of the immune response. Saliva and crevicular fluid contain a wide variety of enzymes and antimicrobial factors that are in contact with the supragingival and subgingival region, including β-defensins (hBDs). hHBDs are non-glycosylated, cysteine-rich cationic peptides produced by epithelial cells with antimicrobial and immunoregulatory effects, thus contributing to maintaining homeostasis in periodontal tissues. The changes in the microbiota and the immune response from a healthy periodontium to gingivitis and, finally, to periodontitis are complex. Their severity depends on a dynamic balance between bacteria associated with plaque, genetic and environmental factors. Recent advances have made it possible to understand the implication of hBDs in the detection, diagnosis, and therapy of periodontal disease and the relationship between periodontitis and other inflammatory conditions. This review aims to describe the effect of hBDs on the immune response and its use as a possible marker of the inflammatory activity of the periodontal disease

Recibido: 14-03-2022 Aceptado: 07-04-2022

INTRODUCCIÓN

La enfermedad periodontal (EP) es un padecimiento inflamatorio en los tejidos de soporte (encía, ligamento periodontal y hueso) de los órganos dentarios (O.D.); típicamente inicia con gingivitis y, al no ser tratada, puede evolucionar a periodontitis crónica con la posibilidad de la pérdida de los O.D [1]. La gingivitis es definida como una condición inflamatoria en un sitio específico de la encía que inicia con un acúmulo de biopelícula alrededor del O.D; clínicamente se caracteriza por la presencia de sangrado, eritema e inflamación gingival [2]. La periodontitis es una patología inflamatoria crónica multifactorial, asociada con una biopelícula disbiótica que produce la destrucción progresiva de los tejidos de soporte del O.D; clinicamente se caracteriza por sangrado al sondeo y presencia de bolsas periodontales [3].

La EP representa un problema de salud pública a nivel mundial debido a su alta prevalencia en muchos países. Así, se estima que en América Latina la prevalencia en países como Brasil, México, Chile, República Dominicana, Argentina, Guatemala y Colombia está entre un 19-96% [4]. El Centro para la Prevención y Control de las Enfermedades en Estados Unidos (CDC) y la Academia Americana de Periodontología han estimado una prevalencia de más del 50% en la población adulta a partir de estudios de casos los cuales consideraron la pérdida de inserción

clínica y la profundidad al sondeo[1]. Por otro lado, estudios epidemiológicos en los cuales se realizaron mediciones continuas de pérdida de inserción clínica mostraron la presencia de enfermedad periodontal avanzada y pérdida dental entre un 10-15% de manera global [1]. En otros estudios se estableció una prevalencia de 64%-96.7% dependiendo del grupo etario, siendo más prevalente en el grupo de más de 65 años [5,6]. Las superficies de la cavidad oral están en contacto constante con una alta variedad de microorganismos que son capaces de formar biopelículas sobre los O. D., la mucosa oral y sobre las prótesis dentales[7]. Una función básica del epitelio gingival es actuar como una barrera mecánica y química en sus diferentes regiones (epitelio oral, del surco y de unión) [8,9], ya que protege a los tejidos de la invasión de los microorganismos [7,10]. El mantenimiento de un estado saludable solo es posible cuando el hospedador mantiene una interacción equilibrada entre la colonización microbiana y los mecanismos de defensa[11]. En este sentido, las células del tejido epitelial oral sintetizan péptidos antimicrobianos (AMPs por sus siglas en inglés) dirigidos contra bacterias Gram-positivas, Gram-negativas, así como levaduras y algunos virus [11,12]. Dentro de los AMPs se encuentran las α y β defensinas presentes en el epitelio gingival [13], glándulas salivales, saliva y fluido crevicular[14]. El objetivo de esta revisión es describir el efecto de las hBDs en la respuesta inmunitaria y su utilización como marcadores de la actividad inflamatoria en la enfermedad periodontal.

Defensinas

Las defensinas son péptidos catiónicos de pequeño tamaño, presentes en un gran número de especies tanto en el reino animal como en el reino vegetal [15]. Fueron reportadas por primera vez en el bovino en 1991 [16] y, posteriormente, en un estudio realizado por Jones y Bevins en 1992 [17] y se describieron en el epitelio intestinal humano con un papel importante en la respuesta inmunitaria innata con actividad antiviral y antibacterial potente [18]. Desde su descubrimiento, han sido intensamente investigadas por su actividad antimicrobiana y sus funciones inmunomoduladoras multifacéticas bajo condiciones fisiológicas y patológicas [19,20]. Es por ello que actualmente se les ha renombrado como péptidos de defensa del hospedador (PDH) [9,15], considerando su función y participación en el proceso de cicatrización [21,22], la homeostasis y el manejo de la diversidad del microbioma.

Las defensinas están constituidas por 16 a 50 aminoácidos, con un peso molecular de 3-4.5 kDa y una alta concentración de arginina; su estructura contiene hojas beta plegadas con 6 residuos de cisteína estabilizadas por tres enlaces disulfuro intramoleculares conservados [19,23,24]. De acuerdo a la topología de los enlaces disulfuro, se clasifican en defensinas alfa, beta y teta (α, β y θ) [19]. Actualmente, se han identificado 6 tipos de α-defensinas en humanos [19,25,26,27] y 31 tipos de β-defensinas, de las cuales, solo las β-defensinas 1, 2, 3, 4, 5 y 6 (hBD-1, hBD-2, hBD-3, hBD-4, hBD-5, hBD-6) han sido aisladas de los tejidos humanos [28]. La θ-defensina se expresa en el reino animal y se encuentra exclusivamente en los monos. Se presentan 6 isoformas en los macacos *rhesus* y la isoforma Defensina Teta Rhesus-1 (DTR-1) es la más abundante [18,29].

β-defensinas

Las β-defensinas más estudiadas son la hBD-1, hBD-2, hBD-3, hBD-4. La hBD-1 es secretada de forma constitutiva; se expresa en el tracto genitourinario y respiratorio [19]. La hBD-1 puede ser modulada por mediadores inflamatorios, mientras que la hBD-2 y hBD-3 son expresadas al ser estimuladas por citocinas proinflamatorias [30-33].

En algunas condiciones como el embarazo se presenta un incremento significativo de hBD-1, lo cual sugiere que otros factores no infecciosos la podrían inducir [34,35]. La hBD-2 se encuentra en el epitelio de las superficies internas y externas del cuerpo humano, tales como la piel y el tracto res-

piratorio e intestinal. Este tipo de defensina es inducida por un estímulo infeccioso [30]. Se ha observado que existe una expresión basal mínima, la cual aumenta enormemente mediante la inducción de patrones moleculares asociadas a patógenos (PAMPs) tales como lipopolisacáridos, peptidoglucanos, lipoarabinomanano o algunas citocinas proinflamatorias como el Factor de Necrosis Tumoral alpha (TNF-α), Interleucina-1beta (IL-1β) e Interferón gamma (IFN-γ) [30,36]. La hBD-3, al igual que la hBD-2, es inducible por estímulos inflamatorios y es detectable *in vitro* posterior a la coestimulación con IL-1 o TNF-α [37]. Este tipo de defensina es de mayor tamaño (5kD) y su mecanismo de acción es similar a las anteriores defensinas. Es posible detectarla en células epiteliales del tracto respiratorio, genitourinario, piel, amígdalas, corazón y músculo esquelético; igual que las otras hBDs tienen un amplio espectro de acción antimicrobiano [34,35,38].

La hBD-4 se expresa en testículo, estómago, útero, tiroides, pulmón, riñón y neutrófilos; además, se expresa en células epiteliales de la cavidad oral [39-41]. Ha sido poco descrita en la EP, aunque podría tener actividad bactericida y fungicida, además de actividad quimiotáctica para monocitos, siendo inducible por algunas citocinas y PAMPs; sin embargo, se inactiva con altas concentraciones de sal. García y col. [11], se refieren a esta defensina como la más potente de todas, aunque esto no ha sido comprobado en otros estudios [36,40,41].

En el epitelio oral normal, el RNAm para hBD-1 y hBD-2 se expresan fuertemente en el estrato espinoso, granuloso y corneo [30,42,43]. En los tejidos gingivales, hBD-1 y hBD-2 se encuentran localizadas en el epitelio del surco, pero no en el epitelio de unión; esto podría deberse a la diferenciación de los queratinocitos en los estratos del epitelio (Fig. 1) [30].

Los patrones de localización para hBD-3 en el epitelio escamoso estratificado, podrían ser los mismos para hBD-1 y hBD-2; sin embargo, hBD-3 ha sido localizada principalmente en el estrato basal del epitelio gingival, por lo que se ha propuesto que podría ser el vínculo entre epitelio gingival y el tejido conectivo, funcionando como enlace entre la inmunidad innata y adaptativa [30,44,45]. De igual manera, las hBDs han sido localizadas en las glándulas salivales y en el fluido crevicular. En este sentido, se ha encontrado la presencia del ARNm de hBD-1, 2 y 3 en la glándula parótida, submandibular y las glándulas salivales menores [25,46].

Las defensinas son activas contra bacterias Gram positivas y Gram negativas, hongos y virus, incluyendo especies de microbios orales como son: *A. actinomycetemcomitans, P. gingivalis, P. intermedia* y *F. nucleatum* [47,48]. La capacidad antibacteriana de las hBDs depende de su concentración en la saliva, siendo para hBD-1 150 ng/mL, hBD-2 450-550 ng/mL, mientras que para hBD-3 es de 730 ng/mL [47]. Estas concentraciones son suficientes para desarrollar su actividad bactericida por sí mismas o actuando en sinergia con otros factores bactericidas presentes, tales como la histatina-5, lactoferrina, mucinas y lisozima [12,42,50].

Se ha estudiado la expresión de estas moléculas en estados patológicos y han sido comparadas en individuos libres de enfermedad; por ejemplo, se ha documentado la elevación de hBD-2 en saliva no estimulada de niños con caries dental de 6-12 años en comparación con niños sanos (Tabla 1) [51,52]. Sin embargo, existen algunas condiciones que pueden afectar la actividad antimicrobiana de las hBDs tales como las altas concentraciones de sodio en la saliva, ya que altera la unión del AMP a la bacteria [48]. En condiciones óptimas las hBDs responden a diversas bacterias, virus, parásitos e infecciones fúngicas [15]. Estos péptidos catiónicos interaccionan con las cargas negativas de las membranas de las bacterias Gram positivas y negativas, de igual forma sobre hongos y virus con envoltura.

El mecanismo de acción biológico lítico de los AMPs que inlcuyen a las hBDs, se da mediante cinco pasos. En primer lugar,

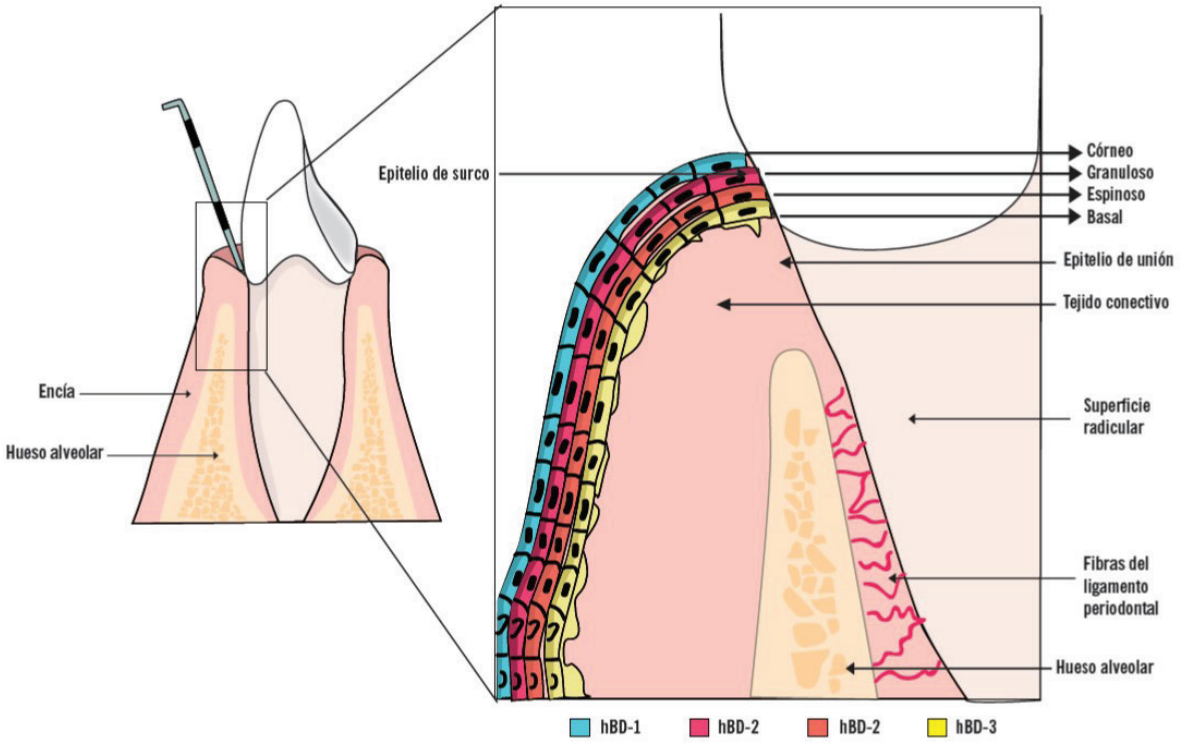

Fig. 1. Expresión de las β-defensinas 1,2 y 3 en el estrato basal, espinoso y granuloso del epitelio en salud periodontal.

Tabla 1
Características de las β-defensinas

Tipo de defensina	Síntesis, expresión y secreción	Concentración en saliva	Activación en tejidos periodontales	Efectos reguladores
β-defensinas (hBDs)	Células epiteliales[10] monocitos, CDs [13]	Salud: hBD-1=150 ng/mL[36] hBD-2=450-550ng/mL[36] hBD-3= 730ng/mL[36]	hBD-1 es secretada constitutivamente[17,24,] hBD-2 y hBD-3 es expresada en infección o inflamación activa[24,29].	Quimioatracción de células T, CDs, macrófagos[9]. Cicatrización de heridas en epitelio[13], Diferenciación, proliferación y migración de queratinocitos[17], mantenimiento de la homeostasis periodontal[18]. Propiedades antimicrobianas e inmunomoduladoras[24], incrementa la expresión de citocinas proinflamatorias[36], promueve la regeneración periodontal[42], neovascularización[43], actividad anti o protumoral[49,53].

los AMPs interactúan con los fosfolípidos de la membrana de la célula del hospedador estableciendo un contacto primario con las células blanco a través de interacciones electrostáticas e hidrofóbicas; en segundo lugar, ocurre un ajuste estructural en la membrana celular (conformación en una estructura de hélice o barril); en tercer lugar, se presenta una acumulación hasta un nivel estequiométrico activo; en cuarto lugar, se presenta una alteración de la membrana a través de la permeabilización o despolarización, ocasionando alteraciones en su función que puede ser transitoria o estable y, finalmente, la muerte de la bacteria [54].

Se han propuesto algunos modelos hipotéticos para la formación de poros en la membrana, tales como el modelo en forma de barril, poro toroidal, alfombra y agregado. Respecto al modelo de barril, al presentarse un aumento en la cantidad y/o expresión del péptido que se une a la membrana, se produce agregación y una transformación conformacional que ocasiona el desplazamiento de los fosfolípidos locales y el adelgazamiento de la membrana [55].

Durante el proceso de penetración dentro de la bicapa fosfolipídica, las regiones hidrofóbicas helicoidales de los péptidos de α-hélice y los péptidos de hoja β-plegada están cerca de las regiones hidrofóbicas del fosfolípido de la membrana, mientras que las regiones hidrofílicas de las hélices peptídicas están hacia dentro; de esta forma, las moléculas se disponen en paralelo para formar la luz central [56].

El mecanismo del modelo de poro toroidal es similar al del modelo de barril, la diferencia es que en el modelo de poro toroidal las hélices peptídicas se insertan en la membrana y se unen a los lípidos para formar complejos de poro toroidal. Los AMPs acumulados localmente en altas concentraciones inducen la deformación de la flexión en las moléculas lipídicas, lo que permite que los péptidos y la cabeza de los grupos lipídicos se inserten dentro del centro hidrofóbico de los lípidos [56].

En el modelo de alfombra, se requieren altas concentraciones de AMPs para formar micelas y la consecuente destrucción de la membrana microbiana [55].

Cuando la concentración del péptido alcanza el umbral, los AMPs cubren la membrana en grupos y provocan su ruptura de manera similar a un surfactante. No se produce la formación de canales, ni inserción de los péptidos en el centro hidrofóbico de la membrana. Este efecto es lo suficientemente potente para inducir la lisis total o parcial de la membrana celular; los AMPs al atravesarla actúan sobre moléculas intracelulares ocasionando la muerte celular (Fig. 2) [57].

En el modelo de agregado, los AMPs se unen a la membrana citoplasmática anióni-

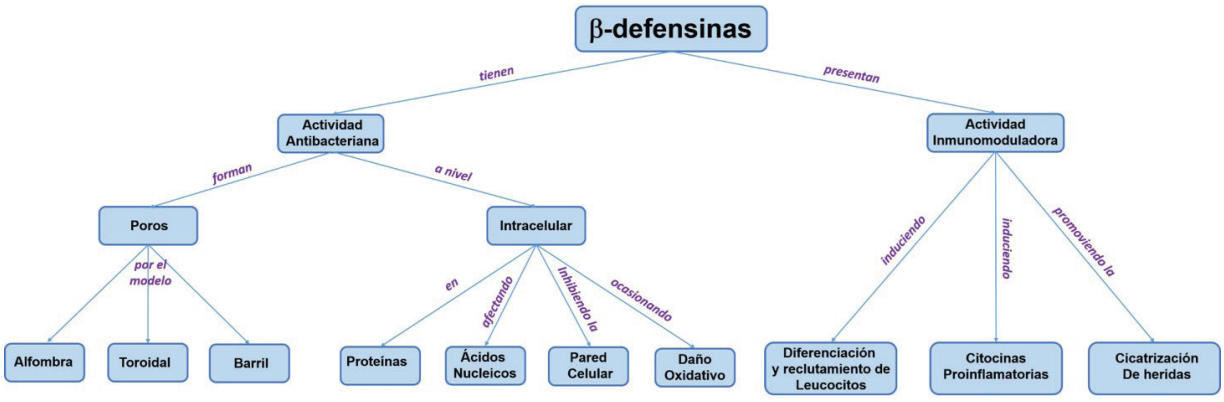

Fig. 2. Clasificación de los mecanismos de acción de los péptidos antimicrobianos y β-defensinas.

ca, obligando a los péptidos y lípidos a formar una micela en un complejo péptido-lípido [58].

A diferencia del modelo de alfombra, los canales formados por AMPs, los iones de lípidos y agua permiten que el contenido intracelular se filtre y provoquen la muerte celular. Estos canales también pueden ayudar a que los AMPs se transfieran al citoplasma y ejerzan su función. Este mecanismo explica por qué los AMPs no solo se dirigen a la membrana citoplasmática, sino que también pueden atravesar la membrana hacia el citoplasma para actuar sobre moléculas intracelulares [57]. De esta forma por su naturaleza anfipática, las hBDs son capaces de insertarse en la membrana fosfolipídica de los patógenos, ocasionando así, la destrucción de la membrana celular [15,59]. De igual manera, las hBDs tienen objetivos intracelulares e interfieren con los procesos metabólicos, incluyendo la síntesis de componentes celulares de vital importancia (Fig. 2) [60].

Por otra parte, existen algunas condiciones que pueden alterar la efectividad de las hBDs como lo es el cloruro de sodio (NaCl). La presencia de NaCl (100mM) reduce la actividad antimicrobiana de hBD-1 y 2 entre un 50-80% hacia *A. actinomycetemcomitans y S. mutans;* sin embargo, la actividad antimicrobiana de hBD-3 no se ha visto afectada (200mM) [48]. En la cavidad oral, las hBDs están expuestas constantemente a los iones en la saliva que contienen 800mM de NaCl. La concentración de NaCl salival es menor de la que se necesita para inactivar a las hBDs; sin embargo, el NaCl no es el único ión presente en la saliva [48]. De hecho, el efecto orquestado de los iones salivales de Sodio (Na^{2+}), Magnesio (Mg^{2++}) y Calcio (Ca^{2++}), pueden afectar parcial o totalmente la función de las hBDs; por lo tanto, es plausible que estos péptidos sean inactivos contra las bacterias fuera de los tejidos, así como en la saliva [48].

En estudios experimentales en ratas libres de gérmenes, se ha observado la producción de precursores de hBDs en ausencia de infección [15]. Estudios *in vitro* en células renales embrionarias, han descrito la expresión de diferentes receptores tipo Toll (TLRs) y hBD-3 mediando la activación de la transcripción del factor NF-κB, que dependen de la expresión de TLR1 y TLR2; esto demuestra que la señalización de TLRs no está restringida de manera única a PAMPs sino que también puede ser iniciado por hBDs [15]. Las hBDs se consideran como un enlace entre la respuesta inmunitaria innata y adaptativa, de tal manera que se ha reportado que la hBD-3 puede intervenir rápidamente estimulando macrófagos vía TLR-4 e impedir la expresión de genes proinflamatorios [15]. De igual manera, inducen la expresión de moléculas coestimuladoras sobre monocitos y células dendríticas (CDs) mieloides dependientes de TLRs, actuando como quimiotáctico para linfocitos T y células dendríticas inmaduras[15]. Las observaciones experimentales y clínicas sugieren que las hBDs son capaces de activar a los macrófagos y favorecer la respuesta proinflamatoria a través de ligandos para TLRs; sin embargo, es necesario ampliar los estudios tanto en animales como en humanos para esclarecer este punto.

A lo largo de la evolución las hBDs han adquirido roles adicionales en un proceso conocido como neofuncionalización, mientras mantienen su rol de defensa original [15]. Se considera que las funciones inmunomoduladoras de las hBDs [61,62] no son mutuamente exclusivas y, por lo tanto, no se deben compartimentalizar sus funciones en una u otra, sino deben ser vistas como parte de la evolución en progreso, como elementos críticos con múltiples roles en funciones complejas contra enfermedades, es por ello que la expresión a través de las mucosas los implica como mediadores fundamentales y centinelas de la homeostasis y la salud [15].

β-defensinas en salud y enfermedad periodontal

La salud periodontal es definida como la ausencia de inflamación clínicamente detectable [63]. La cavidad oral es un ambiente expuesto a una multitud de bacterias; en promedio 800 especies residentes, de las

cuales de 150 a 200 son típicamente encontradas en muchos individuos [1,64]. Estas bacterias son increíblemente complejas y forman comunidades diferentes que viven sobre los O. D., lengua, paladar duro y tejidos epiteliales [64]. La gingivitis inducida por placa y la periodontitis son consideradas como procesos inflamatorios, en los cuales existe una disrupción de la homeostasis provocada por la presencia de una biopelícula, que de manera inicial, se presenta en una forma incipiente y, en la medida que progresa, se convierte en una forma disbiótica hasta ocasionar la destrucción de los tejidos de soporte del O. D., como se ha mencionado con anterioridad [1,65]. La flora bacteriana es controlada inicialmente por el sistema inmunitario innato del epitelio oral, la saliva y el fluido crevicular a través de proteínas y AMPs [65]. Por otro lado, al presentarse un desequilibrio entre la flora oral normal y los patógenos, se forma rápidamente la biopelícula oral sobre la superficie dental; si esto no se corrige, la inflamación gingival progresará posiblemente hacia una periodontitis produciendo pérdida ósea alveolar y finalmente la pérdida dental [66]. La función de los AMPs en la cavidad oral no solo se limitan a controlar el crecimiento bacteriano, sino también a prevenir el crecimiento de la biopelícula. Para tales efectos, bloquea –de manera directa– la aparición de mecanismos de resistencia bacteriana o interfiere con los sistemas de la regulación de la expresión génica, en respuesta a las fluctuaciones en la densidad de población celular de la comunidad bacteriana [67]. Todo ello representa una condición primordial para controlar la EP y mantener la salud periodontal clínica [68]. La inflamación es una respuesta de los tejidos vasculares en la que el sistema inmunitario del hospedador actúa para eliminar la fuente inductora del estímulo inflamatorio [68]. Las infecciones microbianas como la gingivitis inducida por placa y la periodontitis comienzan como una inflamación. Se presenta como una secuencia de eventos bioquímicos coordinados que inician con la rápida migración de leucocitos al sitio de la infección, seguido por un aumento del flujo sanguíneo que transporta diferentes mediadores los cuales controlan el curso de la inflamación [68]. En un inicio, este proceso inflamatorio es benéfico para el hospedador; sin embargo, pueden presentarse mecanismos de resolución inflamatoria ineficientes, que no permiten la eliminación del cuerpo extraño o los mismos patógenos; si esto ocurre, podría darse un proceso inflamatorio crónico [68]. Un gran número de estudios han reconocido a los AMPs, particularmente las hBDs, como moléculas que están implicadas en múltiples actividades proinflamatorias tales como: neutralización de bacterias, quimioataxis y activación de células inmunitarias, neovascularización y cicatrización de las heridas, así como también, actividad anti o pro tumoral [69]. Es importante mencionar que los AMPs interactúan también con la inmunidad innata [70], a través de Receptores de Reconocimiento de Patrones (PRRs), Receptores para quimiocinas (CCRs), el inflamosoma y el sistema del complemento. De esta manera, regula algunos procesos celulares fundamentales en las células del sistema inmunitario, tales como la diferenciación, proliferación y muerte celular programada [68,71]. En épocas recientes, se ha enfatizado que la hBD-2 tiene la capacidad de unirse fuertemente al componente del complemento C1q. Curiosamente, esta proteína no afecta de manera significativa la vía de activación alternativa, pero puede inhibir la vía de activación clásica del complemento y puede estar involucrada en la protección contra la activación descontrolada de la respuesta inmunitaria innata, la interaccion entre hBDs y la respuesta inmunitaria serán explicadas con más detalle, más adelante [72,73].

Las actividades señaladas controlan la inflamación y/o aceleran el proceso de reparación de los sitios infectados, apoyando la función microbicida para la resolución del proceso infeccioso [74]. En este contexto, las hBDs pueden definirse como alarmas o señales de peligro; es decir, moléculas endógenas conocidas como Patrones Moleculares

Asociados a Daño (DAMPs) que se liberan de las células dañadas o moribundas e inician una amplia gama de funciones fisiológicas y fisiopatológicas [68,71]. Así, la hBD-3 crea una disrupción en la biosíntesis de la pared celular uniéndose a regiones ricas en lípidos de la misma, de esta manera, participa rápidamente en el reconocimiento y respuesta a patógenos [75,76].

En diversos estudios se ha demostrado que la expresión de hBDs se correlaciona con el estado inflamatorio del tejido gingival [77-79]. Por ello, con el objetivo de conocer el papel que desempeñan las hBDs en la enfermedad periodontal, se han realizado estudios clínicos en humanos, experimentales *in vivo* e *in vitro*. Dommisch [43], investigó la expresión de las hBDs-1, 2 y 3 en biopsias gingivales de individuos sanos, con gingivitis y periodontitis. En el tejido sano no se encontraron variaciones en la expresión de las hBDs-1, 2 y 3, mientras que en la gingivitis se presentó un aumento en la expresión de la hBD-2 y en la periodontitis hubo una sobre expresión de hBD-2 y hBD-3. Adicionalmente, la expresión de hBD-3 se limitó al estrato basal en condiciones de salud y se extendió al estrato espinoso superficial en condiciones patológicas. Estos datos sugieren que, en la primera etapa del proceso inflamatorio, es decir, en la gingivitis, existe una sobreexpresión de hBD-2, mientras que, en etapas avanzadas de periodontitis, existe una sobreexpresión de hBD-3, además de una alta expresión de hBD-2 [43].

Vandar Seul y cols. [80], estudiaron la expresión del ARNm de las hBD-1 y 2 en los tejidos gingivales de individuos sanos, con gingivitis, periodontitis agresiva y crónica; los resultados demostraron que la expresión génica de la hBD-1 fue baja en gingivitis y periodontitis agresiva, y significativamente alta en el grupo de periodontitis crónica, más que en el grupo control ($p < 0.001$). La expresión del ARNm de hBD-2 en gingivitis fue más baja que en el grupo control y más alta en periodontitis crónica, mientras que en la periodontitis agresiva hubo un incremento en su expresión génica. Por su parte, Ebrahem y col. [81] analizaron la expresión de las hBDs-1, 2 y 3 en fluido crevicular de pacientes con periodontitis agresiva posterior a la terapia periodontal no quirúrgica, se observó un incremento en la frecuencia de la expresión de la hBDs-1 y 3 posterior a la terapia periodontal. Yong y cols. [82], estudiaron la correlación que existe entre periodontopatógenos, parámetros clínicos y expresión de hBD-2; encontraron que la concentración de hBD-2 en fluido crevicular en sitios con EP fue más alta comparada con sitios en salud. Concluyeron que la prevalencia, composición y número de copias de los periodontopatógenos están íntimamente relacionados con la severidad de los datos clínicos de la EP. Brancatisano y cols. [83], evaluaron el nivel de hBD-3 en el fluido crevicular de individuos sanos y con periodontitis; demostraron que, en sujetos con periodontitis, la expresión de hBD-3 fue menor al ser comparado con sujetos sanos. Costa [84], analizó los niveles de hBD-1 en el fluido crevicular en sujetos con y sin periodontitis; encontró que en sujetos con salud periodontal los niveles de hBD-1 fueron más altos que en pacientes con periodontitis crónica, sugiriendo un rol protector de la hBD-1 ante la susceptibilidad a la periodontitis crónica (Figs. 3 y 4). Pereira y cols. [85], evaluaron los niveles de hBD-1 y hBD-2, en el fluido crevicular de individuos con y sin periodontitis; sus resultados demostraron que los individuos con periodontitis presentaron altos niveles de hBD-2 y hBD-3. Sidharthan y su grupo [86], analizaron los niveles de Interleucina-22 (IL-22) y hBD-2 en el fluido crevicular en pacientes sanos, con gingivitis y periodontitis crónica y encontraron que los niveles de hBD-2 fueron significativamente más altos en la periodontitis crónica cuando se comparó con el grupo de gingivitis y sanos ($P<0.001$), indicando el rol de la IL-22 y hBD-2 en la respuesta inmunitaria durante la periodontitis (Tabla 2).

Los datos existentes permiten detectar cambios en la expresión de hBDs en diversos cuadros clínicos dentro de la enfermedad periodontal, evidenciando su participación en

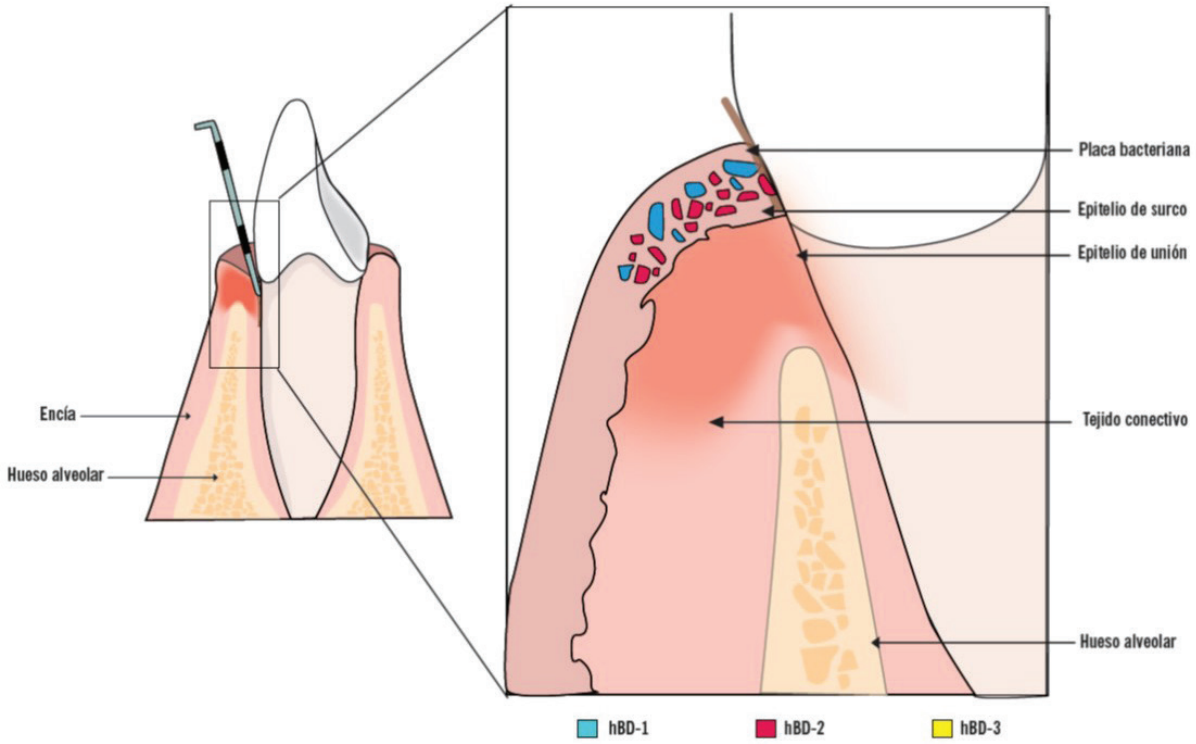

Fig. 3. Expresión de las β-defensinas en el epitelio gingival en gingivitis.

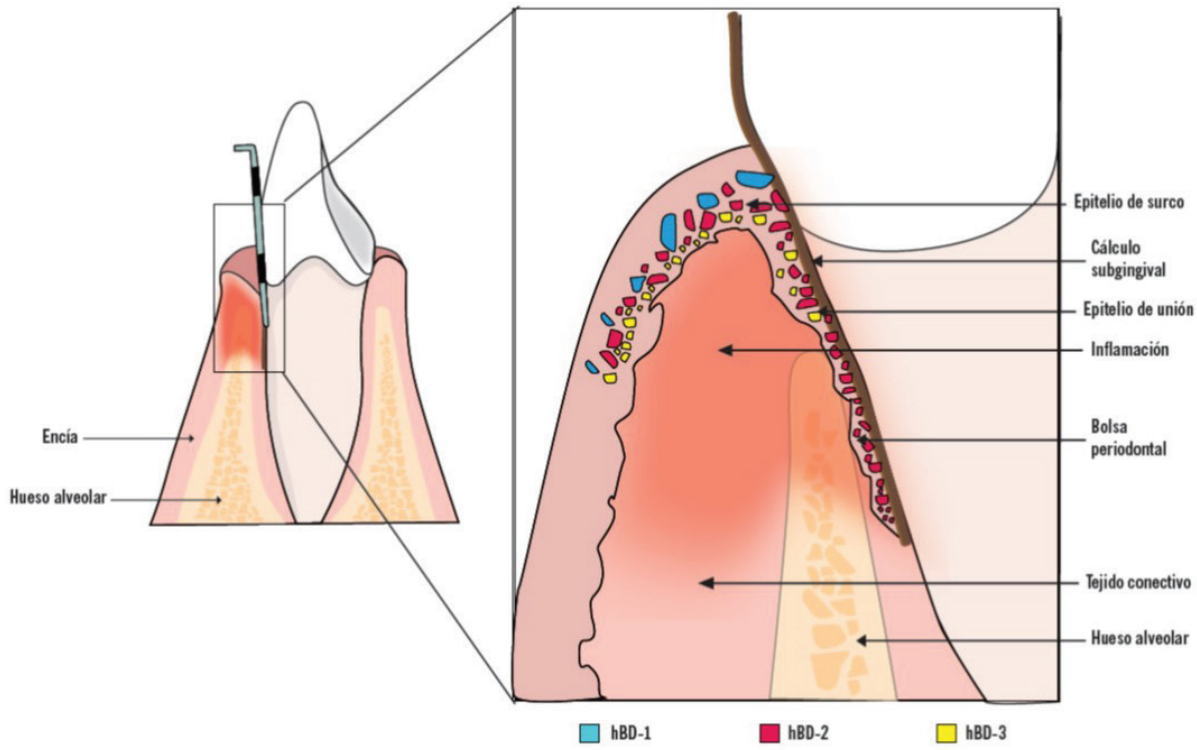

Fig. 4. Expresión de las β-defensinas en el epitelio gingival en periodontitis.

Tabla 2
β-defensinas en la enfermedad periodontal.

Autor	Año	Modelo de estudio y tipo de β-defensina	Enfermedad periodontal estudiada	Resultado del estudio
Dommisch [11]	2005	hBDs-1, 2 y 3	Salud, gingivitis y periodontitis	En salud; niveles de expresión similares para las hBDs-1,-2, y -3. Gingivitis; expresión significativamente alta de hBD-2 comparada con hBD-1. En periodontitis la expresión de hBD-2 fue más alta que hBD-1; sin embargo, hBD-2 fue comparable con hBD-3
Vardar-Sengul [80]	2007	Expresión de ARNm en las hBDs-1 y 2	Salud, gingivitis, periodontitis agresiva, periodontitis crónica	Expresión de genes de hBD-1 fue baja en gingivitis y periodontitis agresiva, y significativamente alta en periodontitis crónica, más que en el control ($p < 0.001$). hBD-2, la expresión del ARNm en gingivitis fue más baja que en el control. El ARNm de la hBD-2 en periodontitis crónica fue más alta cuando se compara con el control. En la periodontitis agresiva incrementó la expresión génica de la hBD-2.
Mohamed [81]	2013	hBDs-1, 2 y 3 En fluido crevicular	Periodontitis agresiva posterior a la terapia periodontal no quirúrgico.	Incrementó en la frecuencia de la expresión de las hBDs-1 y -3 después de la terapia periodontal.
Yong [82]	2015	Expresión de hBD-2.	Periodontitis y salud periodontal	La concentración de hBD-2 en fluido crevicular de sitios con enfermedad periodontal fue más alta comparada con sitios en salud.
Brancatisano [83]	2011	Expresión de hBD-3	Salud y periodontitis	Evaluó el nivel de hBD-3 en fluido crevicular en pacientes sanos y con periodontitis; demostró que en sujetos con periodontitis, la expresión de hBD-3 fue menor al ser comparado con sujetos sanos.
Costa [84]	2018	Niveles de hBD-1, en fluido crevicular	Sanos Periodontitis	Los niveles de hBD-1 en el fluido crevicular fueron más altos en sujetos con salud periodontal comparados con pacientes con periodontitis crónica.
Pereira [85]	2020	Niveles de hBD-1 y hBD-2 en fluido crevicular	Sanos Periodontitis	En periodontitis presentaron altos niveles de las hBD-2 y hBD-3 en el fluido crevicular.
Sidharthan [86]	2020	Niveles de IL-22 y hBD-1 en fluido crevicular	Periodontitis crónica, gingivitis y sanos	Los niveles de hBD-2 fueron significativamente más altos en periodontitis crónica al compararse con gingivitis y los sanos ($P < 0.001$).

la respuesta microbicida e inflamatoria. La identificación de hBDs en fluido crevicular y saliva permite su detección de una manera sencilla, fácil y no invasiva, con el objeto de determinar la actividad inflamatoria de la enfermedad; sin embargo, es necesario considerar algunas situaciones en las que puede encontrarse una disminución de las hBDs y que podrían influir en la interpretación. Una de las responsables es la degradación de hBDs por enzimas proteolíticas producidas por los patógenos periodontales y el hospedador, tales como las proteasas tipo tripsina y las gingipaínas de *P. gingivalis* que degradan a las hBD-1, hBD-2, y hBD-3 [87]. Otras de las enzimas proteolíticas del hospedador que pueden contribuir a la degradación de las hBDs son los miembros de la familia de enzimas cisteín-proteinasa, catepsina B y L, las cuales tienen la capacidad de inactivar a la hBD-2 y hBD-3 en condiciones *in vitro* [88]. Estas enzimas son producidas predominantemente por macrófagos y suelen aumentar en los tejidos gingivales en el inicio y progresión de la periodontitis [88]. Por otra parte, se ha postulado que una mayor participación de la inmunidad adaptativa en comparación con la inmunidad innata durante la enfermedad periodontal, es la responsable en la disminución de la secreción de las hBDs [88]. La expresión de las hBDs, está modulada por el estado inflamatorio de los tejidos; por lo tanto, el incremento de la expresión de las hBDs, ocasiona la presencia de citocinas inflamatorias, por ejemplo, las hBDs-2 y 3 inducen la producción de TNF-α, IL-1 e IFN-γ [89]. En un estudio *in vitro* [90] se observó que las hBD-2 y hBD-3 inducen la maduración de las CDs; las CDs activas son fuente importante de citocinas que reclutan a otras poblaciones celulares, como las células T, las Natural Killer (NK), monocitos y CDs adicionales [91]. En este sentido se ha sugerido que, al pasar de una lesión gingival inicial a una establecida, las funciones antibacterianas de las hBDs son reemplazadas por la inmunidad celular [92], favoreciendo la secreción de IL-8, y el factor estimulador de colonias de macrófagos-1 (MCP-1) y al oncogen que es un regulador de crecimiento (GRO) [92]. Se observó también que las CDs responden de manera diferente a las hBDs-2 y 3; la hBD-2 induce la producción de IL-6 mientras que hBD-3 induce grandes niveles de MCP-1. La inducción de IL-6 e IL-8, pueden ser particularmente importantes ya que son quimiotácticos para neutrófilos y células Th17 [92]. Por otro lado, MCP-1 actúa como quimiotáctico para monocitos y células Treg [93]. Esto indica que las hBDs-2 y 3, inducen de manera selectiva la expresión de citocinas, sugiriendo que las hBDs tienen un rol único en la respuesta inmunitaria al utilizar diferentes receptores para estimular CDs; TLR4, CCR6 y CD91 han sido implicadas como receptores para hBD-2, mientras que TLR1 y TLR2 como receptores para hBD-3 [94]. De tal manera que las hBDs no solo actúan como quimiotácticos para células inmunitarias, sino también inducen patrones de citocinas únicos, que son cruciales en la respuesta inmunitaria hacia bacterias[90]. Otro aspecto importante a considerar es el polimorfismo genético, que se ha asociado con una disminución de hBDs predisponiendo a desarrollar periodontitis, por lo que la relación entre el polimorfismo funcional de los genes de las hBDs y la susceptibilidad a padecer periodontitis es probable, pero necesita ser estudiada a fondo [83].

Los estudios experimentales, por su parte, permiten tener condiciones más controladas que brindan información adicional y valiosa. El desarrollo de la gingivitis clínica se ha estudiado de manera extensa en un modelo denominado gingivitis experimental, descrito inicialmente por Löe y Theilade [95]. Ha sido utilizado en investigación clínica como una herramienta en el estudio de la fisiopatología de la gingivitis en el cual se miden parámetros clínicos (placa e índice gingival) [96] e histológicos en lesiones asociadas a gingivitis para evaluar diferentes grados de inflamación, efectos de agentes farmacéuticos que inhiben la formación de placa bacteriana, así como el efecto en la inmunidad innata. Esto podría ayudar a comprender el

proceso inflamatorio gingival en humanos, en un tiempo corto y con reversión de la inflamación [95]. Esta situación no se podría conocer exactamente en pacientes con gingivitis inducida de manera natural, puesto que no se tiene certeza en qué momento se inicia el proceso inflamatorio y, por tanto, no se puede hacer una correlación entre distintos parámetros clínicos y el inicio de la enfermedad. Sin embargo, una desventaja que tiene el modelo es la duración de la inducción ya que se lleva a cabo en un tiempo no prolongado; por lo tanto, solamente puede ser útil para conocer el fenómeno inflamatorio agudo. Al respecto, se han realizado modificaciones en la fase de inducción, a partir del modelo original de Löe [50], en el que propuso dos fases, la de inducción y la de resolución en un tiempo de 21 días. Posteriormente, ha sido modificado por el modelo experimental de Salvi [97] y Offenbacher [98]. Ramírez-Thomé y cols. [99], realizaron un estudio de 35 días, en el que consideraron 28 días para la fase de inducción y 7 días para la fase de resolución (35 días), a diferencia de los estudios de Dommisch [11,43] que propone un modelo de 14 días y no comprende una etapa de resolución de la gingivitis. Este modelo permite evaluar la expresión de AMPs, por ejemplo, Dommisch y cols. en el 2019 [43] evaluaron la secuencia y expresión diferencial de las hBDs-2 y 3, quimiocina ligando 20 (CCL20), psoriasina/S100A7 (S100A7), y calgranulina A/B (S100A8, S100A9) en biopsias y fluido crevicular, el resultado indicó que hay una expresión elevada de ARNm en los diferentes AMPs explorados. Respecto al análisis de proteínas en muestras de fluido crevicular, encontraron un patrón de expresión similar para hBD-2 y CCL20. Con resultados similares, Dommisch y col. [100] monitorearon la expresión de hBD-2 e IL-8 en fluido crevicular, encontrando elevadas concentraciones de hBD-2 e IL-8. En otro estudio [99] donde se evaluaron parámetros clínicos, microbiológicos y expresión de hBD-1 y 2 en muestras de saliva, no se encontraron diferencias estadísticamente significativas en la expresión de hBD-2; sin embargo, se encontró una mayor concentración de hBD-1, en saliva total en el día 21 y 28 comparado con el día 0. Este aumento coincide con la elevación de hBD-1 durante la fase de inducción (estadísticamente significativa en relación a la hBD-2). Los resultados de diferentes estudios muestran que el comportamiento de los AMPs y que, posiblemente, el tiempo de inducción de la gingivitis genera un sesgo en el momento de comparar los resultados entre los diferentes estudios. En consecuencia, se requieren más estudios controlados con parámetros a medir más uniformes y concretos (Tabla 3).

Los estudios *in vitro*, presentan un papel importante en el esclarecimiento de la participación de las hBDs en la enfermedad periodontal. De acuerdo a Krisanaprakornkit y cols. [75], la incubación de células epiteliales gingivales con *F. nucleatum*, demostró un aumento en la expresión del RNAm de la hBD-2. Vankeerberghen y cols. [101], examinaron la estimulación de las hBDs-1, 2, 3 y 4, utilizando células epiteliales incubadas con bacterias comensales y periodontopatógenas, encontrando que *F. nucleatum* incrementó la secreción de hBD-2 y hBD-3 en las células epiteliales, pero no la hBD-1 y la hBD-4. Por otro lado, *P. gingivalis* no estimuló la secreción de hBD-2 y hBD-4, pero si la secreción de hBD-1 tras 14 horas de incubación, siendo similar el resultado para *A. actinomycetemcomitans* que induce la secreción de hBD-1. La incubación de células epiteliales con *A. actinomycetemcomitans* indujo transitoriamente la expresión de hBD-3, pero después de 3 horas la expresión regresó a los niveles basales. Este cambio dependiente del tiempo en la secreción de las hBDs, puede ser atribuido a la virulencia de las bacterias, ya que invade monocapas de las células epiteliales gingivales en 90 minutos y después se replica intracelularmente. Durante la invasión, *P. gingivalis* inhibe la proliferación y migración de las células epiteliales, modulando el comportamiento de las células del

Tabla 3
β-defensinas en modelos experimentales.

Autor	Año	Modelo de estudio	β-defensina estudiada	Resultados del estudio
Dommisch [43]	2019	Gingivitis experimental	Secuencia y expresión diferencial de diferentes péptidos antimicrobianos.	La expresión de RNAm de los péptidos fue elevada, similar patrón de expresión de hBD-2 y CCL-20 en fluido crevicular.
Ramírez-Thomé [99]	2019	Gingivitis experimental	Parámetros clínicos, microbiológicos que se correlacionan con la expresión de β-defensinas 1 en saliva[51].	Se encontró una mayor concentración de hBD-1 en los días 21 y 28 en comparación con el día 0, no se encontró una diferencia estadística con hBD-2.
Dommisch [100]	2015	Gingivitis experimental	Monitorear la expresión de AMPs e IL-18 en un modelo de gingivitis experimental.	Elevadas concentraciones de hBD-2 e IL-8 en el fluido crevicular.

hospedador para su propio beneficio e inhibiendo también la secreción de las hBDs [8]. Otro estudio [69], evaluó las hBDs-1, 2, 3, 4 y LL-37, y se encontró que las hBDs-2, 3 y 4 pero no hBD-1, estimulan a los queratinocitos humanos para incrementar su expresión génica y la producción de la proteína quimiotáctica para monocitos-1(IP-10), proteína inflamatoria de macrófagos alfa-3, IL-6, IL-10, y RANTES (Tabla 4).

En un estudio donde se evaluó a la hBD-3, en CDs expuestas a hemaglutinina B (HagB) de *P. gingivalis*, se propone que HagB, induce la producción de metaloproteasas de la matriz (MMP) en las CDs y que la hBD-3 en conjunto con HagB, altera los perfiles de inducción de MMP, disminuyendo la respuesta de la MMP-1, 7 y 9; esto sugiere que hBD-3 puede alterar la producción de MMP inducida por antígenos antimicrobianos [102].

CONCLUSIÓN

Las hBDs son péptidos antimicrobianos con múltiples funciones cuyo objetivo principal es el de eliminar microorganismos en los epitelios. Se han identificado diversos tipos de hBDs que producen lisis en las bacterias, además de efectos inmunoreguladores en los leucocitos. De igual modo, se han identificado algunos de los receptores que median la respuesta de las hBDs, en particular para hBD-3 mediado por TLR-4. Sin embargo, aún es necesario esclarecer otros receptores que podrían funcionar en el reconocimiento de la primera señal de activación de la célula, que continúa con la señalización y activación de genes con productos que modulan la respuesta inmunitaria, no solamente para hBD-3 si no también para las otras hBDs. Por otra parte, la evidencia muestra que las hBDs pueden inducir la producción de mediadores proinflamatorios; ahora bien, existen reportes sobre un posible efecto anti-inflamatorio [15,72,73], por lo que deben ser esclarecidas las condiciones, receptores o vías de señalización que puedan favorecer este efecto.

Los diversos estudios en pacientes con EP han mostrado una participación fehaciente de estos AMPs como mecanismos de defensa en esta patología; sin duda, tienen un papel crucial dado que en la mayoría de los estudios realizados se ha encontrado, en general, un aumento en la producción de hBDs. Sin embargo, se requieren

Tabla 4
β-defensinas *in vitro*

Autor	Año	β-defensina estudiada	Modelo de estudio	Expresión
Niyonsaba [69]	2007	hBDs-2, 3, 4	Queratinocitos humanos	Incrementó la expresión de mediadores inflamatorios, proteína-1 quimiotáctica de monocitos, proteína inflamatoria de macrófago -3, RANTES, IL-6, IL-10, e IP-10.
Krisanaprakornkit [75]	2000	hBD-2	*F. nucleatum, P. gingivalis*	+*F. nucleatum*, dispara la expresión del ARNm de la hBD-2. La ++*P. gingivalis* evade el reconocimiento del hospedador.
Vankeerberghen [101]	2005	hBDs-1, 2, 3, 4	Células epiteliales	+*F. nucleatum* incrementa la secreción de hBD-2 y hBD-3. *P. gingivalis*, no estimula la secreción de hBD-2 y hBD-4, incrementa secreción de hBD-1 a las 14 horas de incubación +++*A. actinomycetemcomitans* induce secreción de hBD-1.

+F. nucleatum: Fusubacterium nucleatum , ++P. gingivalis: Porphyromonas gingivalis , +++A. actinomycetemcomitans: Agregatibacter actinomycetemcomitans.

más estudios de forma integral en los cuales se puedan incluir un mayor número de pacientes con diferentes estadios de la EP, para cuantificar las hBDs-1, 2 y 3 en saliva y fluido crevicular en una sola intención, así como al finalizar el tratamiento periodontal. De esta forma se disminuirían los sesgos y se obtendría mayor información sobre el comportamiento de las hBDs durante la enfermedad clínica y en la resolución de la misma; también permitirían la obtención de resultados más homogéneos para analizar la posible utilización de las hBDs como indicador de la actividad inflamatoria en la EP. Adicionalmente, es indispensable continuar con el estudio experimental y clínico de las hBDs-4, 5 y 6, dado que no se tiene información sobre su efecto biológico y sobre la EP. Por otra parte, es importante considerar la degradación de hBDs por enzimas bacterianas y del hospedador y/o su inactivación, como por ejemplo por las concentraciones de NaCl, o en aquellos pacientes que presenten una disminución en las hBDs y que en estos grupos de pacientes los péptidos no podrían funcionar como indicador de la EP.

Como se ha mencionado, el modelo de gingivitis experimental es muy útil para estudiar la inflamación gingival en un corto periodo de tiempo en su etapa inicial. Al respecto, se han realizado diversas modificaciones en el modelo de gingivitis experimental que han aportado información sobre mediadores inflamatorios; sin embargo, se requieren más investigaciones con mayor cantidad de pacientes en quienes se estudien las tres hBDs en saliva y fluido crevicular y añadir una última medición en la etapa de resolución, tal como la describe Ramírez-Thomé y cols. [92]. Así, el modelo originalmente propuesto por Löe y cols., debe ser actualizado para generar un modelo completo, homogéneo, y estandarizado para todos; esto permitiría la comparación de los resultados entre diferentes publicaciones.

Finalmente, las hBDs podrían ser buenas candidatas para ser utilizadas como indicadores de la actividad inflamatoria de la EP; sin embargo, se deben realizar más estudios que permitan enriquecer los ya reportados, así como la publicación de artículos de revisión sistemática y meta análisis, para poder proponer a las hBDs como marcador de la actividad inflamatoria en la enfermedad periodontal.

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Invest Clin 63(4): 435 - 453, 2022 https://doi.org/10.54817/IC.v63n4a09

Angiotensin II and human obesity. A narrative review of the pathogenesis.

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Keywords: obesity; angiotensin II; co-morbidities; adipose tissue; inflammation.

Abstract. Angiotensin II (Ang II) is a hormone and the main effector of the renin-angiotensin system (RAS). This peptide has crucial pathophysiological effects on hypertension, cardiac hypertrophy, endothelial proliferation, inflammation and tissue remodelling through G protein-coupled receptors. The pro-inflammatory role of Ang II has been reported in various inflammatory processes. Obesity is linked to a chronic inflammatory process which in turn is the cause of some of its morbidities. Ang II is related to the comorbidities related to the comorbidities of obesity, which include alterations in the heart, kidney, hypertension and coagulation. In this regard, activation of AT1 receptors by Ang II can induce an inflammatory process mediated by the transcription factor NF-kB, triggering inflammation in various systems that are related to the comorbidities observed in obesity. The aim of this review was to highlight the pro-inflammatory effects of Ang II and the alterations induced by this hormone in various organs and systems in obesity. The search was done since 1990 through Medline, EMBASE and PubMed, using the keywords: *angiotensin II; angiotensin II, obesity; angiotensin II, kidney, obesity; angiotensin II, coagulation, obesity; angiotensin II, inflammation, obesity; angiotensin II, adipose tissue, obesity; angiotensin II, hypertension, obesity; angiotensin II, insulin resistance, obesity; angiotensin II, adiponectin, leptin, obesity; angiotensin II, COVID-19, obesity.* Angiotensin II through its interaction with its AT1 receptor, can induce alterations in diverse systems that are related to the comorbidities observed in obesity. Therapeutic strategies to decrease the production and action of Ang II could improve the clinical conditions in individuals with obesity.

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Angiotensina II y obesidad humana. Revisión narrativa de la patogénesis.

Invest Clin 2022; 63 (4): 435 – 453

Palabras clave: obesidad; angiotensina II; co-morbilidades; tejido adipose; inflamación.

Resumen. La angiotensina II (Ang II) es una hormona y el principal efector del sistema renina-angiotensina (SRA). Este péptido tiene importantes efectos fisiopatológicos en la hipertensión, la hipertrofia cardíaca, la proliferación endotelial, la inflamación y la remodelación tisular a través de receptores acoplados a la proteína G. El papel pro-inflamatorio de la Ang II se ha reportado en diversos procesos inflamatorios. La obesidad está ligada a un proceso inflamatorio crónico que a su vez es causa de algunas de sus morbilidades. Se ha demostrado que la Ang II está relacionada con las comorbilidades de la obesidad, que incluyen alteraciones en el corazón, el riñón, la hipertensión y la coagulación. En este sentido, la activación de los receptores AT1 por la Ang II puede inducir un proceso inflamatorio mediado por el factor de transcripción NFkB desencadenado inflamación en diversos sistemas que se relacionan con las co-morbilidades observadas en la obesidad. El propósito de esta revisión fue destacar el efecto pro-inflamatorio de la Ang II y las alteraciones inducidas por esta hormona en diversos órganos y sistemas en la obesidad. La búsqueda se hizo desde 1990 a través de Medline, EMBASE and PubMed, utilizando las palabras clave: *angiotensina II; angiotensina II, obesidad; angiotensina II, riñón, obesidad; angiotensina II, coagulación, obesidad; angiotensina II, inflamación, obesidad; angiotensin II, adipose tissue, obesidad; angiotensin II, hipertensión, obesidad; angiotensin II, resistencia a la insulina, obesidad; angiotensin II, adiponectina, leptina, obesidad; angiotensina II, COVID-19, obesidad.* La angiotensina II a través de su interacción con su receptor AT1 puede inducir alteraciones en diversos sistemas que están relacionados con las comorbilidades observadas en la obesidad. Estrategias terapeúticas para disminuir su producción y la acción de la AngII pudieran mejorar las condiciones clínicas en individuos con obesidad.

Received: 02-04-2022 Accepted:17-06-2022

INTRODUCTION

Angiotensin II (Ang II) is a hormone derived from the enzymatic digestion of Angiotensin I by the ACE-1 enzyme in the renin-angiotensin system (RAS). In addition to its vasopressor property, this hormone interacts with its AT1 receptor inducing proinflammatory effects through the NF-kB transcription factor and producing gene activation that transcribes proinflammatory proteins and molecules involved in oxidative stress, among others [1-6]. In this way, Ang II induces several inflammatory processes. It has been reported that obesity is highly involved in chronic inflammation [7-9] and that Ang II may play an important role in that inflammation [1, 10-14]. Obesity constitutes a public health problem in view of the associated comorbidities. The comorbidities associated with obesity reach practically all organ systems: type 2 diabetes mellitus, glucose intolerance,

dyslipidemia, hypertension, coronary and peripheral arteriosclerosis and venous insufficiency are some of them. Many of these comorbidities are associated with the inflammatory process of obesity [15]. At the time of the pandemic induced by SARS-CoV-2 (COVID-19), obesity, being an inflammatory process accompanied by several comorbidities, represents a high risk factor for progression to severe disease and death [16]. During COVID-19 there is an increased pro-inflammatory process mediated by Ang II involving high production of cytokines (cytokine storm) [17]. This inflammatory process in a patient with obesity and comorbidities could further exacerbate the already existing inflammation in these patients and determine a severe evolution. In this regard, Ang II has been implicated in the inflammatory process of obesity and its comorbidities [1-6] . Previous studies have shown an increase of serum pro-inflammatory proteins and high expression of AT1 receptor on circulating leukocytes during the onset of the inflammatory process in obesity without co-morbidities [8]. This suggests an initial susceptibility to the action of Ang II in the obesity inflammatory process. Therefore, this review aims to describe the proinflammatory mechanism of Ang II and the possible mechanisms by which Ang II is involved in obesity.

Angiotensin II overview

Angiotensin II is an octapeptide that belongs to the renin–angiotensin system (RAS) and is produced by cleavages of renin forming Ang I that in turn is converted to Ang II by angiotensin converting enzyme-1 (ACE 1). This conversion to Ang II involves the RAS pathway (angiotensin-converting enzyme: ACE); however, the non- RAS pathway (Cathepsin D, Cathepsin G) can also participate in Ang II production. The angiotensinogen is produced in the liver, while renin is produced in the kidney and Ang II in the vascular tissue [2]. ACE2 is another carboxypeptidase that cleaves one amino acid from Ang II leading to the production of the heptapeptide vasodilatory Ang 1–7 [3, 4] and the balance between ACE1 and ACE2 is crucial for controlling Ang II levels [18] . Levels of Ang II can also be regulated by chymase expressed in several tissues (chymase-dependent Ang II-generating system) [19]. These enzymes represent an alternative pathway to ACE in cardiac, vascular, and renal tissue [19, 20]. Other aminopeptidases can cleave Ang II and generate Ang III (2–8) and Ang IV (3–8). Angiotensin III has similar effects to Ang II, although with lower potency (Fig. 1) [5, 21]. Angiotensin IV exerts a protective role by increasing blood flow in the kidney [22] and brain[23]. The presence of RAS components has been observed locally in several organs including the heart [24], kidney [25], brain [26], pancreas [27], and adipose tissues [28], where they have different functions and can operate independently. In addition, a functional intracellular RAS has been identified [29, 30]. The presence of local and intracellular RAS suggests autocrine and apocrine effects of Ang II in different tissues including proinflammatory, proliferative, and pro-fibrotic activities. In this regard, Ang II induces oxidative stress, apoptosis, cell growth, cell migration and differentiation, extracellular matrix remodeling, regulation of inflammatory gene expression and can activate multiple intracellular signaling pathways leading to tissue injury [14, 31]. According to this, the mechanisms of Ang II action can be autocrine, paracrine, and endocrine.

Angiotensin II acts through two distinct G protein-coupled receptors, angiotensin type 1 (AT1, isoforms A and B) and the type 2 (AT2) receptors [6, 32]. AT1A confers actions of Ang II such as blood pressure increase [33], salt retention in proximal tubular cells [34], aldosterone release [35], and stimulation of the sympathetic nervous system in the brain [36]. AT1B regulates blood pressure when AT1A receptor is absent [37]. AT1 and AT2 receptors have counter-regulatory actions in the cardiovascular and renal system [38]. AT2 receptor induces vasodilation and improves artery remodeling and it is upregulated during cardiovascular injury [37]. Angiotensin II

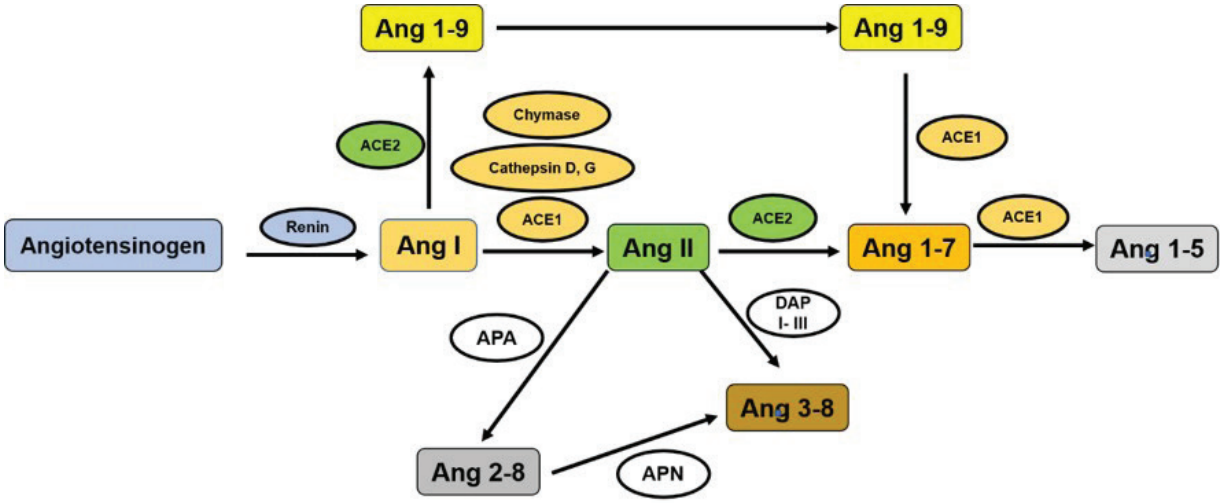

Fig. 1. Renin angiotensin system. The angiotensinogen is transformed into Ang I by the action of the enzyme renin. Ang I is transformed into Ang II by the action of ACE 1, cathepsins D and G or by chymase. In addition to, Ang I can be converted into Ang 1-9 by ACE2 that under the action of ACE 1 converted into Ang 1-7. Ang II can also be converted into Ang 1-7 by ACE2 which under the action of ACE 1 can be transformed into Ang 1-5. Various aminopeptidases can act on Ang II to produce Ang 2-8 and Ang 3-8. ACE 1: angiotensin converting enzyme-1; ACE 2: angiotensin converting enzyme-2; DAP I-III: Dipeptidyl-aminopeptidase I-III; APA: aminopeptidase A; APN: aminopeptidase N; Ang I: angiotensin-I; Ang II: angiotensin-II; Ang 1-5: angiotensin-1-5; Ang 1-7: angiotensin-1-7; Ang 1-9: angiotensin-1-9; Ang 2-8: angiotensin-2-8; Ang 3-8: angiotensin-3-8.

also activates AT1 receptor to induce pro-inflammatory, vasoconstriction, and fibrosis effects; however, activation of AT2 receptor to induce pro-inflammatory effect through NF-kB pathway activation has been also reported [38-40]. AT1 and AT2 receptors also bind Ang III (2-8) and AT4 receptor binds Ang IV (3-8) [41].

Obesity and Inflammation

Obesity is associated with chronic inflammation that increases the risk of developing metabolic diseases, which include hypertension, insulin resistance (IR), altered glucose tolerance, hyperinsulinemia, and dyslipidemia [42]; alterations that together represent the metabolic syndrome (MS). Insulin resistance is a complication of chronic inflammation associated to monocyte/macrophage infiltration and activation of the adipose tissue. This chronic inflammation involves both innate and adaptive immune system [7-10, 43-46] Angiotensin II (Ang II) has been associated to obesity morbidities [10, 47]. During obesity, the precursor of Ang II (angiotensinogen, produced in liver and adipose tissue) is up regulated and related to the growth of adipose tissue and the regulation of blood pressure [11]. Thus, Ang II initiates the activation of an inflammatory process that includes increased oxidative stress, and production of cytokines, chemokines, and growth factors mediated by transcription factor NF-κB activation [1]. In this way, Ang II initiates a chain of inflammatory processes that induce various co-morbidities observed in obesity.

Angiotensin II and adipose tissue

The renin angiotensin system plays a critical role in the pathogenesis of obesity, obesity-associated hypertension, and IR [10]. Angiotensin II can be produced by human adipose tissue; in this regard, angiotensinogen and the enzymes involved in its conversion to Ang II, and both the RAS (renin,

angiotensin-converting enzyme: ACE) and non- RAS (cathepsin D, cathepsin G) pathways are expressed in human adipose tissue. In addition, Ang II receptors are also expressed in adipose tissue suggesting a local role of this hormone in the regulation of adipogenesis, lipid metabolism and in the pathogenesis of obesity [28, 48]. The influence of Ang II on adipocytes is mediated by AT1 and AT2 receptor activation, involving different systems of signal transduction, including Ca 2+ responses, cell proliferation and differentiation, accumulation of triglyceride, adipokine gene expressions and adipokine secretion [49]. Angiotensin II also has anti-adipogenic effect by reducing differentiation of human pre-adipose cells [50]. Therefore, this hormone could be a protective factor against uncontrolled expansion of adipose tissue [51]. This Ang II anti-adipogenic effect has also been observed in omental fat of humans with obesity, involving the participation of the extracellular signal-regulated kinase/1,2 (ERK/1,2) pathway and the phosphorylation of peroxisome proliferator-activated receptor gamma (pPARG) [52, 53]. During this process, the origin of Ang II can be either by RAS or by non-RAS pathways, the latter may be more important in this process [54] . However, in addition to this effect, Ang II can increase triglyceride content and the activities of two lipogenic enzymes (FAS: fatty acid synthase, and GPDH: glycerol-3-phosphate dehydrogenase) in primary cultures of human adipose cells, suggesting control of adiposity through regulation of lipid synthesis and storage in adipocytes [55]. Ang II also regulates the regional blood flow to adipose tissue and the size and number of fat cells [56]. These findings have been confirmed by experimental blocking of Ang II, which directly influences body weight and adiposity (Fig. 2) [57].

The autocrine regulation of Ang II during adipogenesis has also been documented. Angiotensin II can be catabolized in adipose tissues by adipose angiotensin-converting enzyme 2 (ACE2) to form Ang 1-7. The autocrine regulation of the local angiotensin system implies co-expression of Ang II receptors (AT1 and AT2) and Ang 1-7 receptors (Mas) on adipocytes. Activation of the Mas receptor by Ang 1-7 has an effect contrary to the anti-adipogenic effect of Ang II by inducing adipogenesis via activation of PI3K/Akt and inhibition of MAPK kinase/ERK pathways [58]. In this context, the autocrine regulation of the Ang II/AT1-ACE2-Ang 1-7/Mas axis during adipogenesis is capable of producing hormones and cytokines that promote inflammation, lipid accumulation, IR and the components of the RAS, which are activated in the presence of obesity as key obesity-related mechanisms of hypertension and other components of the cardiometabolic syndrome (Fig. 2) [59].

Angiotensin II as a pro-inflammatory agent in obesity

Previous studies have demonstrated the role of Ang II in the inflammation during the obesity. Recently, several experimental studies have shown that Ang II mediates important events of the inflammatory processes [60]. Local activation of RAS and Ang II synthesis increase vascular permeability, mediated by the expression and secretion of vascular endothelial growth factor (VEGF) [61-63], and induce endothelial adhesion molecules expression, such as P and L selectins, vascular cell adhesion molecules-1 (VCAM-1), intercellular adhesion molecules-1 (ICAM-1) and their ligands [64-66], favoring the recruitment of infiltrating inflammatory cells into tissues. In addition, this effect is enhanced by the production of specific cytokine/ chemokines, also mediated by Ang II/ AT1 receptor activation [67-69]. Angiotensin II also promotes endothelial dysfunction through the cyclooxygenase 2 (COX-2) activation, which generates vasoactive prostaglandins and reactive oxygen species (ROS) promoting mitochondrial dysfunction [70-72]. In addition to those effects, a pro-fibrotic effect of Ang II mediated by elaboration of TGF-beta 1, a fibrogenic cytokine responsi-

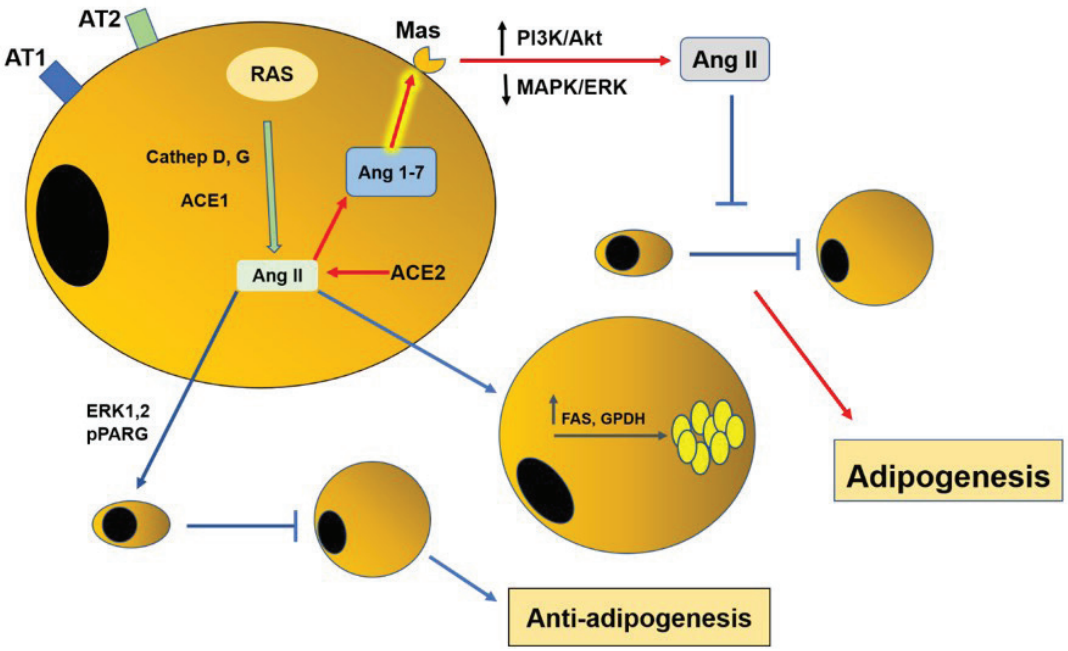

Fig. 2. Adipogenic and anti-adipogenic effects of renin angiotensin system (RAS). Local production of Angiotensin II (Ang II) in adipose tissue, is involved in the regulation of adipogenesis and lipid metabolism. Ang II has anti-adipogenic effect by reducing adipogenic differentiation of human pre-adipose cells involving the participation of ERK(1,2) and the pPARG. Ang II can also increase triglyceride content in adipocytes by activating two lipogenic enzymes, FAS and GPDH. This anti-adipogenic effect of Ang II can be regulated. Ang II can be catabolized by adipose ACE2 to form Ang 1-7 which interacts with Ang 1-7 receptors (Mas) on adipocytes, by activation of PI3K/Akt and inhibition of MAPK kinase/ERK pathways and inducing inhibitory effect in the anti-adipogenic Ang II/AT1, promoting adipogenesis. AT1: Angiotensin II receptor-1; AT2: Angiotensin II receptor-2; RAS: Renin Angiotensin System; Cathep D, G: Cathepsin D, Cathepsin G; ACE1: angiotensin-converting enzyme-1; ACE2: angiotensin-converting enzyme-2; Ang 1-7: Angiotensin 1-7; ERK(1,2): extracellular signal-regulated kinase(1,2); pPARG: phosphorylated peroxisome proliferator-activated receptor gamma; FAS: fatty acid synthase; GPDH: glycerol-3-phosphate dehydrogenase; MAPK kinase/ERK: mitogen-activated protein kinases / extracellular signal-regulated kinases; PI3K/Akt: phosphatidylinositol 3-kinase / protein kinase B.

ble for connective tissue formation and tissular deterioration has been reported [73, 74]. Therefore, Ang II promotes inflammation and tissue injury.

As above explained, Ang II has an important role in the accumulation of body fat during obesity, and obesity is associated with several medical conditions leading to death [75]. In this regard, obesity is associated with the development of hypertension, type 2 diabetes, dyslipidemia, and cardiovascular and renal diseases. Therefore, dysfunction of adipose tissue has been proposed as the cause of visceral obesity-related metabolic disorders, leading to proinflammatory status [76]. In that way, Ang II has been proposed as a promoter of inflammation in obesity associated comorbidities (Fig. 3). Thus, both obesity and hypertension have independently been associated with increased levels of inflammatory cytokines and immune cells within specific tissues, mediated by increased activity of the RAS [12]. Experimental studies have shown association of obesity, Ang II and proinflammatory processes. In this context, consumption of a high-fat diet by mice induces proinflam-

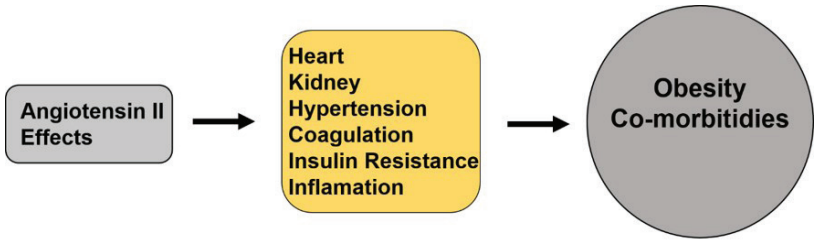

Fig. 3. Pro-inflammatory effects of Angiotensin II (Ang II) on obesity. Ang II is intimately linked to obesity and its pro-inflammatory effects are involved in their co-morbidities, such as insulin resistance, hyperinsulinemia, impaired glucose tolerance, dyslipidemia, and hypertension.

matory responses in the hypothalamus and the subfornical organ, which are known to regulate blood pressure and energy balance accompanied by increased RAS activity [12]. The sensitization of Ang II-elicited hypertension by a high-fat diet in rats was reported, mediated by upregulation of the brain RAS and central proinflammatory cytokines [77]. Exogenous administration of Ang II to rats led to increased monocyte chemoattractant protein-1 (MCP-1) expression in epididymal, subcutaneous and mesenteric adipose tissue. In vitro studies in Ang II treated adipocytes showed increased MCP-1 production mediated by AT1 receptor and NF-kB-dependent pathway, suggesting a link between obesity, Ang II and inflammation [78]. Angiotensin II increases inflammation and endoplasmic reticulum stress in adipocytes via AT1 receptor and mediated by the miR-30 family, -708-5p and/or -143-3p [79]. In a rat model of obesity hypertension, induced by a high-fructose diet, downregulation of adipose RAS, reduced inflammation in adipose tissue and improved obesity hypertension [80].

The initial factors involved in generating the inflammatory events in human obesity remain unclear. Analysis regarding to the presence of Ang II and its AT1 receptor on individuals with obesity, without co-morbidities, showed similar serum levels of Ang II and decreased production of Ang II by circulating mononuclear cells (CMC) in both, individuals with obesity and controls. However, an increased number of CMC expressing the AT1 receptor was observed in individuals with obesity; suggesting that Ang II production does not play an important role in the early period of obesity inflammatory alterations. However, high expression of Ang II receptors may be a preliminary step, with further cellular activation by Ang II [8]. These findings may represent different functional periods of Ang II in the obesity inflammatory events to induce co-morbidities, in which, the initial Ang II pro- inflammatory effects are not found, but in advanced stages of the obesity complications, this molecule may have deleterious effects [8]. In this regard, blocking of Ang II in overweight and patients with obesity associated with multiple comorbidities results in a substantial increase in adiponectin levels and improved IR [13]. Fig. 4 shows in a general way the inflammatory, vasopressor and insulin resistance effects of Ang II.

Angiotensin II and kidney in obesity

Angiotensin II has been implicated in renal damage during obesity. Obesity as a proinflammatory state is associated to kidney diseases and to the development and progression of chronic kidney disease (CKD). Angiotensin II plays an important role in renal damage during obesity. In this regard, increased Ang II contributes to hyperfiltration glomerulomegaly, by altering renal hemodynamics, and subsequent focal glomerulosclerosis [14, 31]. In addition, the imbalance between increased Ang II and the ACE2/Ang 1-7/Mas receptor axis, contributes additionally to renal injury in obesity.

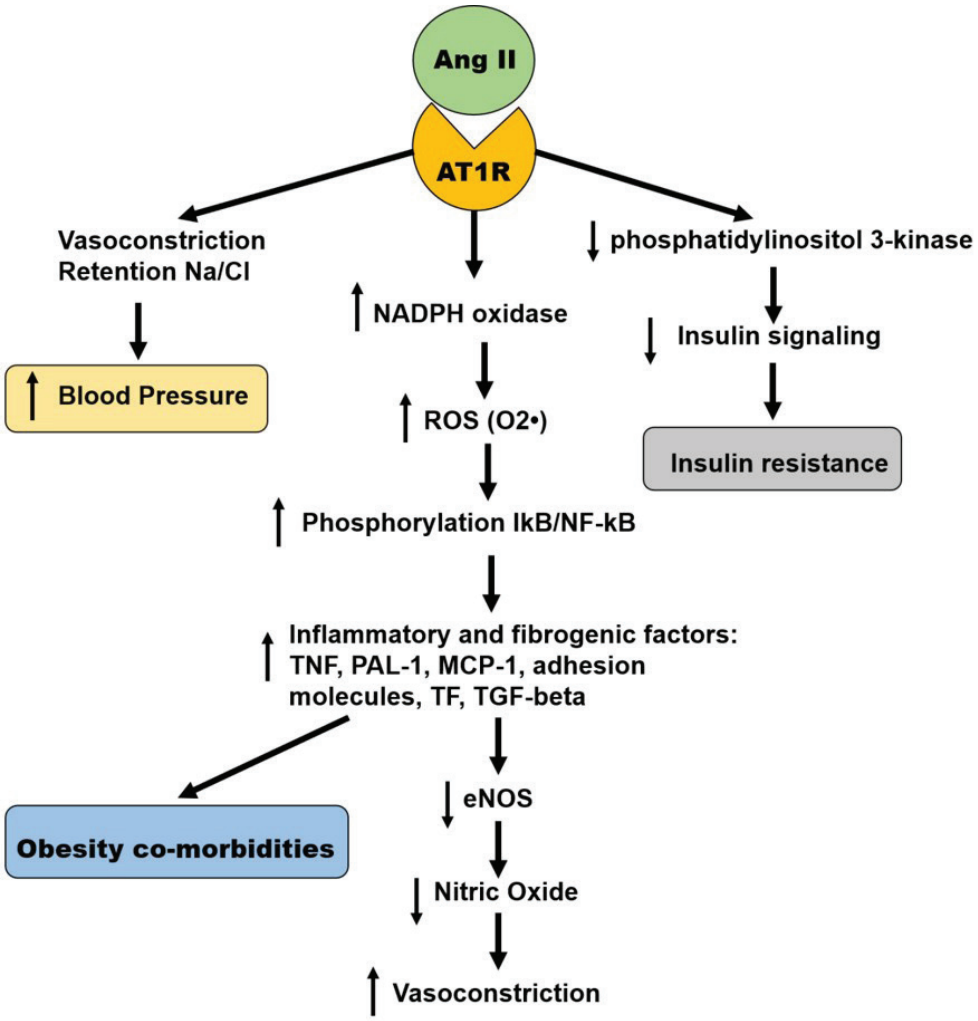

Fig. 4 Interaction of Angiotensin II (Ang II) and its receptor (AT1R) during obesity. After activation of the AT1receptor by Ang II, a series of intracellular processes are initiated that lead to increased blood pressure, insulin resistance and production of co-morbidities during obesity. ROS: reactive oxygen species; TNF: tumor necrosis factor; TF: tissue factor; Pal-1: plasminogen activator inhibitor-1; MCP-1: monocyte chemoattractant protein1; TGF-beta: transforming growth factor-beta; NADPH: reduced form of nicotinamide-adenine dinucleotide phosphate; IkB: inhibitor kB; NFkB: nuclear factor kB; eNOS: endothelial nitric oxide synthase.

The therapeutic blocking of the production or action of Ang II improves the adverse effects on the kidney during obesity [14]. Angiotensin II regulates sodium/fluid homeostasis and blood pressure in the kidney mediated by the activation of AT1 receptors. In obesity, an exaggerated action of Ang II has been implicated in the increased renal sodium retention and the resetting of the pressure natriuresis leading to hypertension. These effects could be related to increased plasma insulin levels observed in obesity which upregulate both AT1 and AT2 receptors in the kidney [81]. During obesity and azotemia, the oxidative stress stimulates synthesis of Ang II, which in turn increases tumor growth factor-beta (TGF-β) and plasminogen activator inhibitor-1 expressions, inducing glomerular fibrosis. Furthermore, in these patients, local synthesis of angiotensinogen by

adipocytes, leptin activation of sympathetic nervous system, and hyperinsulinemia contribute to the development of hypertension and CKD in obesity [82]. Renal abnormalities induced by Ang II in the obesity may also be related to the effects of oxidative stress on the large conductance, Ca (2+)-activated K (+) channels in podocytes. In addition, Ang II induces podocyte apoptosis [83]. Other possible cause of renal failure is the excessive leptin production in patients with obesity. Leptin induces dysfunction of intrarenal vessel endothelium and microalbuminuria and increases circulating endothelin-1. These disorders in obesity can be improved by administration of Ang II receptor blockers [84]. Experimental results show that obesity augments vasoconstrictor reactivity to Ang II in the renal circulation of the Zucker rat, providing insight into early changes in renal function that predispose to nephropathy in later stages of the disease [85]. Considering the data exposed, Ang II has a relevant role in the renal damage during obesity mediated by structural, hemodynamic, and biochemical alterations (Fig. 5).

Angiotensin II and heart in obesity

Previous studies have reported that during obesity, Ang II is able to induce cardiac and arterial damage. Visceral adipose tissue plays a key role in the metabolic and cardiovascular complications in obesity. Angiotensin II may be involved in modulating both intracardiac lipid content and lipid metabolism-related gene expression, in part via AT1 receptor-dependent and pressor-independent mechanism [86]. Angiotensin II and catecholamines may induce increased G protein-coupled receptor kinase 2 (GRK2) lev-

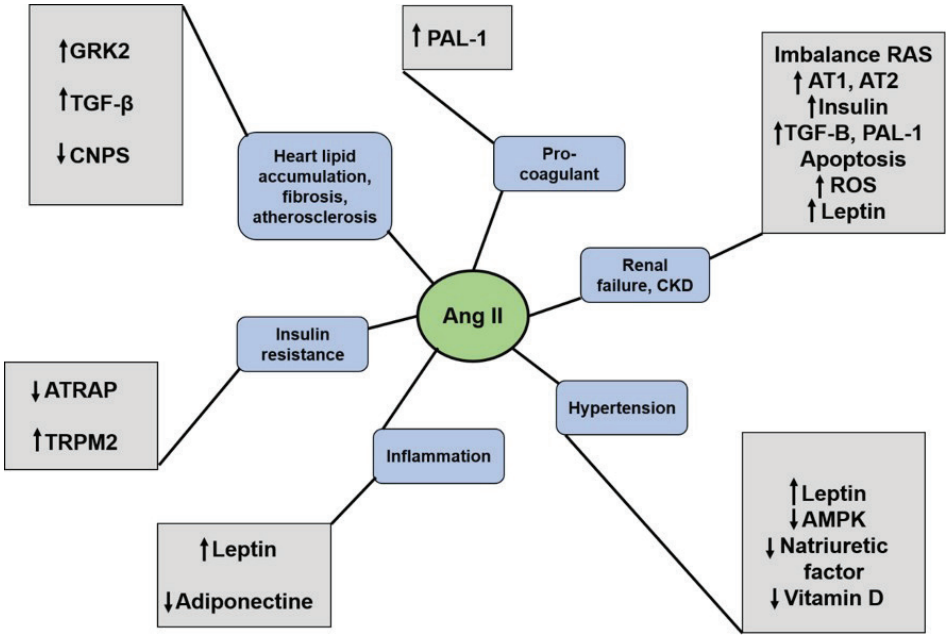

Fig. 5 Effects of Ang II on various organs and systems in obesity. Angiotensin II is involved in various effects on the heart, kidney, insulin resistance, hypertension, coagulation, controlling leptin and adiponectin levels, and inflammatory processes among others during obesity. Ang II: Angiotensin II; AT1: Angiotensin II receptor-1; AT2: Angiotensin II receptor-2; RAS: Renin Angiotensin System; TGF-B: Transforming growth factor-beta; ROS: Reactive oxygen species; AMPK: Adenine monophosphate -activated protein kinase; PAL-1: plasminogen activator inhibitor-1; CKD: Chronic kidney disease; GRK2: G protein-coupled receptor kinase 2; CNPS: cardiac natriuretic peptide system; ATRAP: AT1 receptor-associated protein; TRPM2: Transient receptor potential melastatin 2.

els in diverse cardiovascular cell types. This can explain the contribution of increased GRK2 levels to altered cardiovascular function and remodeling in obesity [87]. Lipid accumulation in the heart is associated with obesity and may play an important role in the pathogenesis of heart failure. Myocyte steatosis can increase the fibrotic effects of Ang II mediated by the activation of TGF-β signaling and increased production of ROS [88]. The visceral adiposity and cardiometabolic complications are linked to IR, sympathetic nervous system, RAS and cardiac natriuretic peptide system (CNPS). Renin angiotensin system and CNPS are antagonistic systems on sodium balance, cardiovascular system, and metabolism. As expressed, RAS activity is increased in patients with obesity; however, CNPS, which induces natriuresis and diuresis, reducing blood pressure, and has powerful lipolytic activity is found reduced in these patients. Thus, reduced CNPS effects coupled with increased RAS activity have a central role in increased obesity cardiovascular risk [89]. During obesity increased serum Ang II and TNF-α levels have also been reported. Experimental data have shown that these two peptides may interact to exacerbate myocardial ischemic/reperfusion injury [90]. Atherosclerosis is a complex, chronic disease that usually arises from the converging action of several pathogenic processes, including obesity, hypertension, hyperlipidemia, and IR. The capacity of Ang II to induce atherosclerosis and cardiovascular injury has been reported in both human and animal studies [91]. Despite the harmful Ang II effects on the heart, some of its metabolites (Ang 1-7) may have beneficial cardiovascular and metabolic effects when Ang 1-7 interacts with the Mas receptor (Fig. 5) [92].

Angiotensin II and hypertension in obesity

Angiotensin II associated with obesity represents a high risk factor of hypertension in obese individuals. Angiotensin II is associated with obesity hypertension [47]. Arterial hypertension represents one of the comorbidities observed in obesity and the renin-angiotensin-aldosterone system is an important effector [93]. Obesity can increase the risk of hypertension and cardiovascular disease in individuals born prematurely, since obesity may increase the prematurity-associated imbalance in the RAS [94]. During obesity increased levels of circulating leptin which can increase sympathetic nerve activity and raise blood pressure have been reported. This leptin induced hypertension is mediated by up-regulation of central RAS and proinflammatory cytokines [95].

Angiotensin II is also capable of suppressing AMPK activity in the kidney, leading to sodium retention, enhanced salt-sensitivity, and hypertension [96]. In addition, obese hypertensive men have a relative natriuretic peptide deficiency and inadequate RAS suppression, one of the mechanisms by which obesity leads to hypertension [97]. Obesity and vitamin D deficiency have both been linked to augmented activity of the tissue RAS. In obesity, decreased levels of 25-hydroxyvitamin D are associated with increased vascular sensitivity to Ang II leading to hypertension (Fig. 5) [98].

Angiotensin II and insulin resistance in obesity

One of the obesity morbidities is the loss of insulin sensitization of the insulin receptor. Previous studies have demonstrated the relationship of Ang II with insulin resistance. Insulin is a hormone that allows glucose to enter cells in different tissues which also reduces blood glucose. Insulin resistance is defined clinically as the inability of a known quantity of exogenous or endogenous insulin to increase cellular glucose uptake and utilization in consequence blood glucose levels increase. Obesity, sedentarism, and family history of diabetes are some of risk factors for IR [99]. Previous studies have shown that Ang II is an important promoter of IR and diabetes mellitus type 2 [100]. Angiotensin II-induced IR is suppressed by increased AT1 receptor-associated protein (ATRAP) in

adipose tissue, hyperactivity of AT1 receptor induced by Ang II decreases ATRAP and could be related to IR [101]. Other mechanism, as the action of redox-sensitive transient receptor potential melastatin 2 (TRPM2), has been proposed. TRPM2 is a positive regulator of Ang II-induced adipocyte IR via Ca^{2+}/CaMKII/JNK-dependent signaling pathway. Inhibition of TRPM2 improves insulin sensitivity induced by Ang II in adipose tissue [102]. Blocking of the AT-1 receptor also improves IR mediated by Ang II and changes induced by adiponectin in patients with diabetes mellitus [103]. These data suggest that Ang II increases the action of TRPM2 with subsequent IR production (Fig. 5).

Angiotensin II and adiponectin, and leptin in obesity

Angiotensin II may modulate the action of leptin and adiponectin in obesity. There is evidence that dysregulation in the production of adipocytokines is involved in the development of obesity-related diseases. Two important adipocytokines, leptin and adiponectin are associated to obesity, IR, increased risk of coronary heart disease and type 2 diabetes mellitus. Decreased levels of the anti-inflammatory adiponectin, while increased levels of proinflammatory cytokine leptin associated with obesity, IR and endothelial dysfunction have been reported [104]. Leptin and adiponectin have opposite effects on inflammation and IR. Leptin upregulates proinflammatory cytokines such as TNF-α and interleukin-6 associated with IR, type 2 diabetes mellitus and cardiovascular diseases in the obesity [104]. Angiotensin II and its metabolites acting on AT1 receptor can stimulate leptin production in human adipocytes. This effect is mediated by an extracellular-signal-regulated kinase 1 and 2-dependent pathway [105] and can increase the pro-inflammatory activity of leptin during obesity. On the other hand, leptin decreases Ang II-induced vascular effect by blocking the vasoconstrictor action of Ang II and inhibits the Ang II-induced increase in intracellular Ca (2+) in vascular smooth muscle cells [106]. Plasma concentrations of adiponectin correlated negatively with a vast majority of risk factors, such as obesity, type 2 diabetes, glucocorticoids, testosterone, and hyperlipidemia, suggesting a protective role of adiponectin. Blocking of RAS increases plasma adiponectin suggesting a role of Ang II in decreased levels of adiponectin. Supporting this, Ang II infusion decreased plasma adiponectin and adiponectin mRNA in adipose tissue. Angiotensin II also interacts with adiponectin in their target cells. In this regard, the misbalance between adiponectin, Ang II, and IR in endothelial cells can determine the endothelial dysfunction in metabolic syndrome and obesity [107-109]. There is evidence indicating that adiponectin has reno-protective effects and protects against the development of albuminuria induced by Ang II in obesity (Fig. 5) [110], suggesting that Ang II-decreased effect on adiponectin may be involved in renal damage.

Angiotensin II and coagulation in obesity

Angiotensin II may alter the fibrinolytic system in obesity. The connection between obesity and hemostasis disorders is well established. The inhibition of fibrinolysis in the obesity, associated to increased plasma inhibitor, plasminogen activator inhibitor-1 (PAI-1) has been documented [111, 112]. PAI-1 is the main inhibitor of the fibrinolytic system and was recently shown to be produced by adipose cells. Obesity is associated with an increased production and release of PAI-1 protein. Angiotensin II and its metabolites promote PAI-1 production and release by human fat cells and may contribute to the impairment of the fibrinolytic system typical for obesity. AT1 receptor blockade reduces basal and abolishes Ang II-stimulated PAI-1 release from human adipocytes (Fig. 5) [111, 112].

CONCLUSION

The renin angiotensin system and especially Ang II are highly involved in the patho-

logical events that occur in obesity. Angiotensin II through its interaction with its AT1 receptor can induce alterations in diverse systems that are related to the comorbidities observed in obesity. Therapeutic strategies to decrease the production and action of Ang II could improve the clinical conditions in individuals with obesity.

Limitations of the review

The reports studied for this review are only based on the concept of obesity referring to individuals with a BMI greater than 30, with or without morbidities.

ACKNOWLEDGEMENTS

We thank Instituto de Investigaciones Clínicas Dr. Américo Negrette, Facultad de Medicina, Universidad de Zulia, Maracaibo, Venezuela.

Funding

This manuscript has no financial support.

Declaration of conflict of interest

The authors have no competing interests to declare that are relevant to the content of this article.

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Author's contributions

JM-S and ER conceived the subject matter and contributed to the design of the work. JM-S, ER, RV and AP contributed to the acquisition, analysis, or interpretation of data for the work. JM-S and ER wrote the original draft. JM-S, ER, RV and AP critically revised the first draft. All authors approved the final version for all aspects of work ensuring integrity and accuracy.

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