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EDITORIAL
Plagio, similitudes e inteligencia artificial en la evaluación de trabajos científicos.

Los comités editoriales de diferentes revistas científicas, han establecido para la evaluación de los trabajos de investigación, el plagio, las similitudes con otros trabajos y el uso de "inteligencia artificial (IA)", como criterios de rechazo de los mismos, sin la valoración previa de los expertos. Plagio se define de acuerdo al Diccionario de la Real Academia Española (RAE), como la acción de "copiar en lo sustancial obras ajenas, dándolas como propias" [1]. En otras palabras, es la apropiación indebida del trabajo intelectual de otra persona, presentándolo como propio, sin la debida atribución. En el caso de las instrucciones a los autores de revistas de habla inglesa, "Plagiarism is defined as presenting another person's work as your own, whether it's text, ideas, data, or images, without proper attribution. This includes self-plagiarism, where an author reuses their own previously published work without proper citation" [2]. "Originality and plagiarism: The authors should ensure that they have written entirely original works, and if the authors have used the work and/or words of others, that this has been appropriately cited or quoted" [3]. En otras palabras, la citación de la referencia, eliminaría el concepto de plagio en el trabajo de investigación. En el caso de los trabajos de investigación científica y llevándolo al extremo de copiar exactamente lo que dicen los autores en otros trabajos, no se está cometiendo plagio ya que se define a través de las referencias, que es propiedad intelectual del autor referenciado. De hecho, muchas veces no se colocan las palabras exactamente como el autor las dice, sino la idea que expresa, y se colocan las referencias en cuestión. Algunas revistas colocan en sus indicaciones a los autores, que poseen el sistema para determinar el "Plagiarism", pero lo reportan como similitudes con otras publicaciones, sin considerar la presencia de la referencia, que en tal caso no representaría un plagio. Además de evaluar la cantidad y calidad del contenido copiado, se ha establecido en estos programas la siguiente pregunta: ¿Se ha beneficiado el autor de la habilidad y el criterio del autor original? [3]. En general la respuesta es sí, ya que contribuye a la realización de algunas de las secciones del trabajo de investigación, pero no representa un plagio al ser referenciado, de hecho, el autor obtiene aumento en la citación de su trabajo. Sin embargo, el "Plagiarismo" es considerado una grave falta y un motivo de rechazo del trabajo, sin ser enviado a los revisores. Esta situación es manejada por programas preestablecidos en la red [4].

En referencia al concepto de similitudes, que posiblemente esté vinculado al plagiarismo, se establece que todo lo que se reporte en un trabajo, que tenga una similitud con trabajos de otros autores, con el internet, etc., es motivo de su no aceptación para el envío a revisores. Entre las similitudes pueden estar incluidos el nombre de los autores, su afiliación, líneas o párrafos que se parecen a otras publicaciones, etc. Obviamente esto también es producido por programas preestablecidos [4]. Aquí se llega a la posición ilógica de validar como similitudes e incluirlas en el porcentaje de similitudes,

los nombres de los autores, adscripciones y abreviaciones ampliamente aceptadas en otras publicaciones, etc., Estos programas[4] representan las similitudes como números que no definen lo que se presenta como similar. En estos casos, un alto porcentaje de "similitudes", también representa un impedimento para el envío de los trabajos de investigación a los revisores.

En referencia al uso de la IA, existen programas que hacen este análisis y reportan el porcentaje generado por inteligencia artificial [4]. El correcto uso de la IA puede ser de beneficio para las publicaciones científicas, siempre y cuando la inteligencia natural, establezca el orden de lo reportado por la IA. La IA puede ayudar a los investigadores en el proceso de la escritura del manuscrito, mejorando la redacción y el lenguaje, pero no en la interpretación, análisis y conclusiones lógicas de los resultados encontrados, lo cual deben ser realizado por los investigadores. La búsqueda de investigaciones o reportes, usando palabras clave de un determinado aspecto de la Ciencia, muchas veces es infructuosa si se usa la IA, ya que frecuentemente, solo toma en cuenta algunas de las palabras clave, y origina reportes incompletos o no adecuados para el fin que se busca. Los autores deben indicar en sus manuscritos, cuando los someten a revisión, el uso de la IA, para mostrar transparencia y obviamente la IA, no debe de ser citada como un autor [5]. El uso de la IA de manera adecuada, no debe ser un obstáculo para el envío del manuscrito a investigadores expertos en la materia de estudio.

En conclusión, el uso de programas que indiquen porcentajes de plagio (basados en el concepto de plagio), similitudes o IA, sin previo análisis de la calidad del manuscrito, representa una actitud editorial basada en guías de nueva tecnología, que no determinan la calidad de los manuscritos, y no debe ser una condición que impida el envío de trabajos de investigación a los revisores que analizan la calidad del reporte.

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Plagiarism, similarities and artificial inteligence in the evaluation of scientific papers

The reliance on programs that measure percentages of plagiarism (based on the concept of plagiarism), similarities, or AI content—without a thorough analysis of the manuscript's quality—reflects an editorial approach that prioritizes new technology guidelines over the actual quality of the work. Such measures should not serve as a barrier to the submission of research papers for review by qualified reviewers who can assess the quality of the report.

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A preliminary investigation of the association between KRAS, NRAS, and BRAF mutations and colorectal cancer in Turkish patients.

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Keywords: NRAS; BRAF; KRAS; colorectal cancer.

Abstract. Somatic mutations in the GTPase RAS protein family and the downstream serine-threonine kinase BRAF are predicted to be key driver mutations in colorectal carcinogenesis by disrupting critical control points in cell cycle regulation. In our study, we aimed to investigate the relationship between KRAS, NRAS, and BRAF mutations in colorectal cancer (CRC) samples and corresponding clinicopathological data. This retrospective study included 64 CRC patients who were evaluated for KRAS, NRAS, and BRAF mutations in our department between 2022 and 2024. The findings were evaluated according to the age, gender, tumor localization in the colon, and histopathological subtype of the patients in whom the mutation was detected, and the relationships between these variables were analyzed using the chi-square test. KRAS mutations were detected at 29.6%, NRAS mutations at 3.1% and BRAF mutations at 1.6%. No significant relationship was found between mutation rates and the patients' age, gender and colon localization. Our study demonstrated that mutations in KRAS, NRAS, and BRAF were not associated with the age, sex, and tumor location of CRC patients. The data presented are preliminary findings, and more research is needed to evaluate the clinical and pathological impact of these mutations on colorectal cancer progression and outcomes.

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Una investigación preliminar sobre la asociación entre mutaciones KRAS, NRAS y BRAF en cáncer colorrectal en pacientes Turcos.

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Palabras clave: NRAS; BRAF; KRAS; cáncer colorrectal.

Resumen. Se predice que las mutaciones somáticas en la familia de las proteínas GTPasa RAS y la serina-treonina quinasa BRAF son mutaciones clave en la carcinogénesis colorrectal al interrumpir puntos de control críticos en la regulación del ciclo celular. En este estudio, el objetivo fue evaluar la relación entre las mutaciones KRAS, NRAS y BRAF en muestras de cáncer colorrectal (CCR) y datos clinicopatológicos. Este estudio retrospectivo incluyó a 64 pacientes con CCR que fueron evaluados para mutaciones en KRAS, NRAS y BRAF en nuestro servicio entre 2022-2024. Los hallazgos se evaluaron según la edad, el sexo, la localización del tumor en el colon y el subtipo histopatológico de los pacientes en los que se detectó la mutación, y se analizaron las relaciones entre ellos mediante la prueba de chi-cuadrado. Las mutaciones en KRAS se detectaron en un 29,6%, en NRAS en un 3,1% y en BRAF en un 1,6%. No se encontró una relación significativa entre las tasas de mutación y la edad, el sexo y la ubicación del colon de los pacientes. Nuestro estudio demostró que las mutaciones en KRAS, NRAS y BRAF no se asociaron con la edad, el sexo y la localización del tumor de los pacientes con CCR. Los datos presentados son nuestros hallazgos preliminares y se necesita más investigación para evaluar el impacto clínico y patológico de estas mutaciones en la progresión y los resultados del cáncer colorrectal.

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INTRODUCTION

The incidence rates of colorectal cancer (CRC) in adults (under 55 years of age) have been increasing by 1–2% annually. In the late 1990s, CRC was the fourth leading cause of cancer death in both men and women under 50. However, it has since escalated to become the leading cause of cancer death in men and the second leading cause in women within this age group [1]. CRCs are the second leading cause of death from malignancy, and more than half of CRCs become metastatic [2]. Despite the substantial impact this disease has on both quality of life and the healthcare system, data regarding the molecular analysis of biomarkers in patients diagnosed with CRC is limited [3].

Several oncogenes, particularly mutations in RAS and BRAF, have a significant influence on colorectal carcinogenesis. These mutations lead to the activation of the mitogen-activated protein kinase signalling pathway, which is crucial for cell proliferation, differentiation, and survival. Activation of this pathway promotes tumorigenesis by driving uncontrolled cell growth and facilitating processes such as angiogenesis and metastasis. Understanding the role of these oncogenes provides insight into the mecha-

nisms of colorectal cancer and identifies potential therapeutic targets [4].

Identifying abnormalities in the KRAS, NRAS, and BRAF genes is crucial for accurately qualifying CRC patients for treatment with anti-epidermal growth factor receptor (EGFR) monoclonal antibodies [5]. Oncogenic RAS mutations are found in approximately 50–55% of metastatic colorectal cancer (CRC) cases, with regional variations in prevalence. Importantly, these mutations serve as negative predictive markers for response to monoclonal antibodies that target the epidermal growth factor receptor (EGFR). This means that patients with RAS mutations are less likely to benefit from EGFR-targeted therapies, making it crucial to screen for these mutations when determining treatment options for metastatic colorectal cancer (mCRC). Identifying RAS status can help guide more effective and personalized treatment strategies [4]. BRAF mutations occur in approximately 5–17% of CRC cases, with the BRAFV600E mutation being the most prevalent. In mCRC, the existence of the BRAFV600E mutation not only serves as a negative predictive marker for response to EGFR-targeted MoAbs but is also associated with a markedly poor prognosis. This mutation often indicates a more aggressive disease course and resistance to conventional therapies, highlighting the need for alternative treatment strategies and close monitoring of affected patients. Understanding the implications of BRAF status is crucial for optimizing management and enhancing outcomes in mCRC. The status of tumor localization and CC laterality in determining prognosis and guiding treatment remains controversial. It is thought to be due to differences in histological, genetic and immunological features between the left and right colon [6,7]. In the present study, we hypothesized that somatic mutations in the GTPase RAS protein family and the downstream serine-threonine kinase BRAF could interfere with essential checkpoints in cell cycle regulation, acting as significant driving mutations in colorectal carcinogenesis. Our goal was to assess the presence of KRAS, NRAS, and BRAF mutations in clinicopathological features of CRC samples in our hospital.

PATIENTS AND METHODS

Study population and design

For this retrospective study, KRAS, NRAS, and BRAF mutations were analyzed in 64 CRC biospecimens at the Bakırçay University Faculty of Medicine Laboratory. Patients were characterized by age, gender, and tumor location, with rectal and sigmoid cancers being the most prevalent. The histological classification of tumors by the WHO was used as a guide to assign the histological subtypes (classical adenocarcinoma and mucinous adenocarcinoma) and tumor location (colon segment other than rectum, and rectum) [8].

To conduct the study, ethics committee approval was obtained from the İzmir Bakırçay University Non-Interventional Clinical Research Ethics Committee on 03.04.2024, with decision number 1681 and research number 1661.

DNA extraction was conducted using the QIAamp formalin-fixed paraffin-embedded (FFPE) kit (Qiagen, USA). Tissue samples were collected at the time of diagnosis for colon and rectal cancer. FFPE primary tumor samples from 64 CRC patients between January 2022 and December 2024 were retrospectively reviewed. Mutation analysis was performed with the KRAS/BRAF, NRAS, and BRAF Mutation Analysis Kit for Real-Time PCR (Diatech Easy, Italy). The tests targeted the most common mutations in exon 2 (codons 12 and 13), exon 3 (codons 59 and 61), exon 4 (codons 117 and 146) of the KRAS and NRAS genes, and exon 15 (V600E) of the BRAF gene.

Statistical Analysis

For the statistical analysis, a chi-square test was used to examine the associations

between mutated genes (KRAS, NRAS, and BRAF) and various clinicopathological variables, including age, gender, and the specific localization of the tumor in the colon. The analysis was conducted using SPSS version 23.0, and p-values of ≤ 0.05 were deemed statistically significant, contributing to a better understanding of their implications in colorectal cancer.

RESULTS

The study included patients aged 33 to 79 years, with a mean age of 62.54 and a median age of 64. Among them, 41 (64%) were male and 23 (36%) were female (Table 1). In our study, no mutations were found in 20 patients (31.2%). KRAS exon 2 mutation was observed in 29.7% of cases, while other RAS mutations were found in 3.1% of cases, and the BRAFV600E mutation was seen in 1.6%. One patient had KRAS mutations at positions 12 and 13, and the other had KRAS mutations at position 61 and NRAS mutations at position 12.

Table 1. Mutation profile of colorectal cancer patients stratified by age and gender.

	Wild type *n = 44*	Mutant *n = 20*	*p*
Age <64	26 (76.5%)	8 (23.5%)	
Age ≥64	18 (60%)	12 (40%)	0.125
Female	18 (78.3%)	5 (21.7%)	
Male	26 (63.4%)	15 (36.6%)	0.172

No associations were found between the age and gender of CRC patients and the frequency of KRAS, NRAS, and BRAF gene mutations. Notably, these mutations were more frequently diagnosed in men (64%) than in women (36%). Table 1 shows the distribution of mutation presence in patients with colorectal cancer according to age and gender.

A total of 47 cases had tumor localization identified. In 14 cases, the tumors were located in the left colon, while 33 cases were localized in the right colon. In the case of the BRAFV600 mutation, tumor localization could not be determined. The distribution of RAS and BRAF mutation values in colorectal cancer patients according to age, sex and localization is shown in Tables 2, 3, and 4.

DISCUSSION

Colorectal cancer is the third most common type of cancer in both sexes in Turkey. According to the 2024 official cancer statistics, 152,810 (7.6%) of the 2,001,140 cancer cases diagnosed in 2019 were CRC. Reports indicate a significantly higher incidence in men (53.3%) compared to women [1,4]. In our study, most of the patients were male (64.1%), consistent with national cancer statistics [1].

Our study aimed to evaluate the presence of somatic mutations in the GTPase RAS family of proteins (KRAS and NRAS) and the downstream serine-threonine kinase BRAF in CRC samples. It is hypothesized

Table 2. Distribution of KRAS, NRAS and BRAF mutations by age in colorectal cancer patients.

	KRAS			NRAS			BRAF		
	Wild type *n=45*	Mutant *n=18*	Total *n=63*	Wild type *n=62*	Mutant *n=2*	Total *n=64*	Wild type *n=63*	Mutant *n=1*	Total *n=64*
Age <64	24 (38%)	8 (12.7%)	32 (50.7%)	33 (51.6%)	1 (1.6%)	34 (53.2%)	34 (53.1%)	0 (0%)	34 (53.1%)
Age ≥64	21 (33.3%)	10 (15.9%)	31 (49.2%)	29 (45.3%)	1 (1.6%)	30 (46.9%)	29 (45.3%)	1 (1.6%)	30 (46.9%)
p		0.360			0.722			0.469	

Table 3. Sex-based frequency of BRAF, KRAS, and NRAS mutations in colorectal cancer patients.

	BRAF			KRAS			NRAS		
	Wild type *n=63*	Mutant *n=1*	TOTAL *n=64*	Wild type *n=46*	Mutant *n=18*	Total *n=64*	Wild type *n=62*	Mutant *n=2*	Total *n=64*
Female	23 (36%)	0 (0%)	23 (36%)	33 (51.5%)	4 (6.2%)	23 (35.9%)	22 (34.2%)	1 (1.7%)	23 (35.9%)
Male	40 (62.5%)	1 (1.5%)	41 (64%)	27 (42.2%)	14 (21.9%)	41 (64%)	40 (62.5%)	1 (1.5%)	41 (64%)
p		0.414			0.126			0.641	

Table 4. Distribution of KRAS and NRAS mutation frequencies according to tumor location in colorectal cancer.

	KRAS		NRAS		
Tumor Location	Wild type *n=31*	Mutant *n=16*	Wild type *n=45*	Mutant *n=2*	Total *n=47*
Colon	11 (23.4%)	3 (6.4%)	13 (19.4%)	1 (1.5%)	14 (20.9%)
Rectum	20 (29.9%)	13 (19.4%)	32 (47.8%)	1 (1.5%)	33 (49.3%)

that these mutations might disrupt critical checkpoints in cell cycle regulation and serve as key driving factors in colorectal carcinogenesis. The detection rates of KRAS, NRAS, and BRAF mutations in our cohort of 64 CRC patients were 29.7%, 3.1%, and 1.6%, respectively. Consistent with the findings of Mosaferi et al. [8], our study also observed that the majority of KRAS mutations were located in exon 2, while most NRAS mutations were found in exon 3 [8]. In line with the findings of Khoshnoudi et al. [2] and Mosaferi et al. [8], our study found no significant association between KRAS mutation status and age or gender.

The prevalence of KRAS mutations in our study aligns with findings from the literature, which consistently reports that KRAS mutations occur in approximately 40% of CRC cases [9]. These mutations are critical in terms of poor prognosis and resistance to targeted therapies, such as anti-EGFR agents. [10,11] The relatively lower frequency of NRAS (3.1%) and BRAF mutations (1.6%) observed in our study is consistent with their reported rates in the literature, where BRAF mutations are often associated with more advanced disease stages and poorer outcomes [12,13]. Our findings showed that mutation rates were not significantly associated with the patients' age, gender, or colon localization.

A study by Yamauchi et al. [14] showed that the frequency of BRAF mutations increased from the rectum to the ascending colon and decreased towards the cecum. Ekmekciu et al. [15] reported that KRAS mutations were predominantly located in the right colon, but no significant differences were observed in age, stage, or histopathological subtype. Bylsma et al. [16] found BRAF and RAS mutations more frequently in right-sided colon cancers, consistent with Ekmekciu et al. [15]. In addition, Bylsma et al. reported that BRAF and RAS mutations were more common in young patients [16]. Our findings showed that mutation rates were not significantly associated with patients' age, gender, or colon localization.

We would like to emphasize that the present study has limitations related to the cohort size. The limitations of the study included that some cases in the study group were obtained from consultations with external centers, while others were obtained from colonoscopic biopsy materials. For these reasons, the relationship between prognostic markers for tumor staging and mutation results could not be compared in our study. This study is essentially an exploratory, preliminary study, which requires definitive, confirmatory values. Our findings highlight the complex interaction between molecular subtypes, clinical and histopathological features in CRC and the need for further investigation of the underlying mechanisms driving these relationships. In the future, stratifying patients according to molecular subtypes may provide advantages in personalized treatment approaches.

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Conflict of interest

The authors declare that there is no conflict of interest associated with this article.

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Author contributions

TD and SST designed the study. TD analyzed and interpreted data and searched the literature. TD and GE drafted the manuscript. All authors reviewed the manuscript and approved the final version.

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Analysis of prognostic factors and construction of a risk model for patients with acute cerebral infarction treated with dual antiplatelet therapy after optimal hyper thrombolytic time window.

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Keywords: brain infarction; platelet aggregation inhibitors; combination; aspirin thrombolytic therapy; time factors; risk factors.

Abstract. The objective was to identify potential risk factors for the prognosis of dual antiplatelet therapy in patients with acute cerebral infarction (ACI) treated beyond the optimal time window to prevent thrombolysis, and to construct a nomogram to evaluate such risk. The clinical data of 300 ACI patients treated outside the optimal hyperthrombolytic time window and admitted to our hospital from January 2020 to May 2024 were analyzed retrospectively. The association between potential risk factors for poor prognosis after dual antiplatelet therapy was tested by logistic regression. A nomogram was constructed to evaluate the risk of poor prognosis based on the results. The area under the receiver operating characteristic (ROC) curve (AUC) was used to evaluate the ability of the model to differentiate types of prognoses. A calibration curve evaluated the consistency of the model, and the fitting of the model was evaluated by the Hosmer-Lemeshow (HL) test. Of the 300 patients, 52 (17.3%) had a poor prognosis. Old age, hypertension history, elevated homocysteine, elevated fibrinogen level and carotid artery stenosis were risk factors associated with poor prognosis in patients with ACI. A nomogram was built based on these risk factors. The AUC, calibration curve, and HL test demonstrated that the selected model was statistically capable of discriminating between good and poor prognosis. In conclusion, advanced age, a history of hypertension, elevated homocysteine, elevated fibrinogen, and carotid artery stenosis are risk factors associated with a poor prognosis for patients with ACI treated beyond the optimal time window. If validated, the nomogram based on these five risk factors could be used to distinguish between cases with poor prognosis and those with good prognosis among these patients.

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Análisis de factores pronósticos y construcción de un modelo de riesgo en pacientes con infarto cerebral agudo tratados con doble terapia antiplaquetaria después del tiempo óptimo para evitar hiper trombólisis.

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Palabras clave: infarto cerebral; inhibidores de la agregación plaquetaria; terapia combinada; aspirina; terapia trombolítica; factores de tiempo; factores de riesgo.

Resumen. El objetivo fue identificar factores asociados con el pronóstico de pacientes con infarto cerebral agudo (ICA) tratados con doble terapia antiplaquetaria después del periodo óptimo para evitar trombólisis, y construir un modelo de riesgo en forma de nomograma. Se analizaron retrospectivamente los datos clínicos de 300 pacientes, tratados después del tiempo hipertrombolítico, que fueron ingresados en nuestro hospital entre enero del 2020 y mayo del 2024. Los factores asociados con el mal pronóstico tras la doble terapia con antiplaquetas se analizaron con regresión logística. De acuerdo a los resultados, se construyó un nomograma para modelar el riesgo de un mal pronóstico. Se utilizó el área bajo la curva de la característica operativa del receptor (ABC) para evaluar la capacidad del modelo para diferenciar entre tipos de pronósticos. La consistencia del modelo se evaluó mediante una curva de calibración y el ajuste del modelo se evaluó mediante la prueba de Hosmer-Lemeshow (HL). De los 300 pacientes, 52 (17,3%) tuvieron un mal pronóstico. El análisis logístico mostró que la vejez, los antecedentes de hipertensión, el nivel elevado de homocisteína, el nivel elevado de fibrinógeno y la estenosis de la arteria carótida fueron factores de riesgo asociados con un mal pronóstico de los pacientes con ICA. Se construyó un nomograma basado en estos factores. La ABC, la prueba de HL, y la curva de calibración mostraron que el modelo seleccionado es estadísticamente capaz de distinguir entre buenos y malos pronósticos. Como conclusión, la vejez, los antecedentes de hipertensión, el nivel elevado de homocisteína, el nivel elevado de fibrinógeno y la estenosis de la arteria carótida son factores de riesgo asociados con un mal pronóstico en pacientes con ICA tratados después del período óptimo. Si es validado, el nomograma basado en estos cinco factores de riesgo podría ayudar a distinguir entre casos de buenos y malos pronósticos en estos pacientes.

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INTRODUCTION

Stroke is a primary chronic noncommunicable disease that seriously endangers health. According to data from the World Health Organization, the annual death rate of stroke accounts for 10.7% of the global death rate [1]. In China, stroke has become one of the major diseases causing death and disability and is the first cause of death and disability in adults, with five characteristics: high incidence, high disability rate, high mortality rate, high recurrence rate and high economic burden [2]. Stroke can cause neurological impairment of patients, seriously affect their long-term prognosis, and lead to the decline

of cognitive function and daily living ability [3], and some patients may have residual limb dysfunction [4]. The most common clinical stroke is ischemic stroke, that is, ACI, which is caused by a disturbance in the brain blood supply, and the main clinical manifestations are ischemic necrosis of localized brain tissue and nerve function deficit. Currently, the treatment of ACI primarily includes intravenous thrombolysis, intravascular intervention, and antiplatelet therapy. For ACI patients within 4.5 hours after onset, intravenous thrombolysis is the preferred method for vascular recanalization [5]. However, a considerable number of patients have insufficient understanding of acute cerebral infarction and miss the time window for effective treatment. When patients miss the optimal time for thrombolytic therapy, clinical treatment options are minimal. Therefore, dual-antiplatelet therapy (i.e., aspirin combined with antiplatelet drugs such as clopidogrel) within the hypercoagulable time window has become an important therapeutic method [6]. By inhibiting platelet aggregation and preventing thrombus formation and expansion, dual antiplatelet therapy can improve brain blood flow and promote nerve function recovery[7]. However, its therapeutic effect and prognosis are influenced by numerous factors, and responses vary significantly among patients. At present, there are relatively few studies on the prognostic factors of dual-antiplatelet therapy in ACI patients after the optimal hyperthrombolytic time window. In addition, most existing risk assessment models are based on thrombolytic therapy, and the risk model for hyperthrombolytic time-window dual therapy is not perfect, which is why it is challenging to meet the clinical demand for individualized prognosis assessment. Therefore, the purpose of this study was to analyze the prognostic factors affecting dual-antiplatelet therapy in ACI patients beyond the hyperthrombolytic time window, and to construct a nomogram which, if validated, will provide clinicians with an individualized prognostic assessment tool, optimize the treatment plan, improve the treatment effect, and reduce the disability rate and mortality.

MATERIALS AND METHODS

General information

Three hundred and ten patients outside the hyperthrombolytic time window, admitted to the Department of Neurology at our hospital from January 2020 to May 2024, were included as initial samples. However, ten patients with incomplete clinical data were excluded, resulting in a total of 300 patients meeting the inclusion criteria. Inclusion criteria: (1) Meet the diagnostic criteria of ACI [8]. (2) Admission within 24h after onset but beyond the time window of intravenous thrombolysis. (3) Aspirin combined with clopidogrel was given within 24 hours after admission. (4) Age ≥18 years. Exclusion criteria: (1) Patients with drug allergies. (2) Patients with cerebral hemorrhage, acute infection and malignant tumor. (3) Patients with coagulation dysfunction or thrombocytopenia. (4) Incomplete clinical data. This study was reviewed and approved by the Tianjin Hospital Ethics Committee.

Treatment method

Patients received routine basic interventions, including improving circulation, controlling blood pressure and blood sugar levels, and stabilizing plaque. At the same time, Clopidogrel (Sanofi Pharmaceutical Company, approval number: H20171238, specification: 75 mg x 7 tablets) was administered orally at a dose of 75 mg once daily. Aspirin (Bayer Healthcare Co., LTD., Sinopol: HJ20160685, specification: 100mg ×30 tablets) 100mg/ time, 1 time/day, orally. A course of treatment lasts for seven days. The patient persisted in the treatment for two courses.

Data collection

Through consulting electronic medical records, clinical data of patients were collected, including age, gender, history of hypertension, diabetes, smoking history,

drinking history, atrial fibrillation history, stroke history, homocysteine (HCY) and fibrinogen (Fib), high density lipoprotein, C-reactive protein (CRP) levels and carotid artery stenosis.

Outcome measurement

The primary measure of this study was the prognosis of patients with ACI. The prognosis was assessed 14 days after the onset of the disease, and the National Institutes of Health Stroke Scale (NIHSS) score was used to evaluate the prognosis of patients after treatment. The NIHSS score ranges from 0 to 42, with higher scores indicating more severe nerve damage. The score is as follows: 0 to 1: normal or nearly normal; 1-4 scores: mild stroke/minor stroke; 5~15 points: moderate stroke; 15-20 points: moderate to severe stroke; Scores 21-42: severe stroke. In this study, a score of 0 to 4 was defined as a good prognosis, and a score of 5 to 42 was defined as a poor prognosis.

Statistical method

The SPSS 23.0 statistical software was used for data analysis. Measurement data were expressed as mean ± standard deviation ($\overline{x}\pm sd$), and inter-group comparison was performed using the t-test of two independent samples. Statistical data were expressed as cases and percentages [n (%)], and the χ^2 test was used for comparison between groups. Logistic regression analysis was used to test for associated factors. $p<0.05$ was considered statistically significant. A sample of 70% of the 300 cases was selected for training to construct the model, while the remaining 30% was selected to evaluate the model's performance. The statistically significant factors were introduced into RStudio software to construct a nomogram. The model's discrimination power was evaluated using the ROC curve and calibration curve. The Hosmer-Lemeshow analysis was used to evaluate the goodness of fit of the nomogram model, and a p greater than 0.05 indicated good consistency.

RESULTS

General data comparison

According to the prognosis assessment, 52 of the 300 patients (17.3%) had a poor prognosis. There were significant differences in age, history of hypertension, homocysteine, fibrinogen, and carotid artery stenosis between the good prognosis group and the poor prognosis group (all $p<0.05$). No significant difference was observed in the comparison of other indicators (all $p>0.05$), as shown in Table 1.

Multifactor analysis

Variables with statistical significance in univariate analysis were included as independent variables, and whether there was a poor prognosis was taken as the dependent variable (yes =1, no =0). The variable assignment table is shown in Table 2. The results showed that old age, history of hypertension, elevated homocysteine level, elevated fibrinogen level, and carotid artery stenosis were risk factors for poor prognosis of ACI patients treated with dual antiplatelet therapy (Table 3).

Construction of a nomogram of prognostic factors in ACI patients

Taking 210 patients in the training set as samples, based on the results of multifactor analysis, the above five statistically significant factors were included in the risk assessment, and a column-line risk model was established (Fig. 1). The specific prediction model formula is as follows: Logit (P)= -42.356 + 0.378 × (Age) + 1.391 × (Hypertension) + 1.503 × (Carotid stenosis) + 1.471 × (Homocysteine) + 1.396 × (fibrinogen). The corresponding scores can be obtained by projecting the corresponding Points of each variable to the "points" axis. The corresponding scores can be added together, and the total scores obtained can be used to asses prognosis.

Verification of the nomogram model

To further verify the predictive efficiency of the model, ROC curves for the training set

and the test set were plotted separately (Fig. 2). The model had a high prediction accuracy in both the training set and the test set, with an ACU of 0.963 (95%CI: 0.920~1.000) and 0.969 (95%CI: 0.927~1.000), respectively. The Hosmer-Lemeshow test showed a good fit (χ^2 =14.933, p = 0.060). The calibration curve (Fig. 3) demonstrates good agreement between the prediction probabilities of the training set and the test set. In addition, the decision curve shows a significant increase in the net benefit of the nomogram (Fig. 4).

Table 1. Comparison of clinical data between the two groups.

Factors	Good prognosis group (n=248)	Poor prognosis group (n=52)	c^2/t	*p*
Age (y)	61.31±3.58*	68.27±5.26*	9.111	<0.001
Gender				
female	104(41.94)**	20(38.46)**	0.214	0.644
male	144(58.06)**	32(61.54)**		
Diabetes				
No	166(66.94)**	29(55.77)**	2.356	0.125
Yes	82(33.06)**	23(44.23)**		
Hypertension				
No	172(69.35)**	23(44.23)**	11.927	0.001
Yes	76(30.65)**	29(55.77)**		
Smoking history				
No	130(52.42)**	27(51.92)**	0.004	0.948
Yes	118(47.58)**	25(48.08)**		
Drinking history				
No	133(53.63)**	28(53.85)**	0.001	0.977
Yes	115(46.37)**	24(46.15)**		
Atrial fibrillation history				
No	207(83.47)**	39(75.00)**	2.088	0.148
Yes	41(16.53)**	13(25.00)**		
Stroke history				
No	168(67.74)**	29(55.77)**	2.733	0.098
Yes	80(32.26)**	23(44.23)**		
Carotid stenosis				
No	184(74.19)**	20(38.46)**	25.223	<0.001
Yes	64(25.81)**	32(61.54)**		
Homocysteine (μmol/L)	6.56±0.98*	8.65±1.56*	9.268	<0.001
High density lipoprotein (mmol/L)	1.31±0.21*	1.26±0.24*	1.506	0.133
C-reactive protein (mg/L)	5.39±0.99*	5.49±1.06*	0.681	0.497
Fibrinogen (g/L)	2.80±0.59*	3.29±0.77*	4.344	<0.001

Note: Data are represented as* mean ± standard deviation, and as ** n (%).

Table 2. Factor assignment table.

Factor	Assign
Age (y)	Original input
Hypertension	0=No,1=Yes
Carotid stenosis	0=No,1=Yes
Homocysteine (μmol/L)	Original input
Fibrinogen (g/L)	Original input

DISCUSSION

Previous studies have confirmed that factors such as inflammation, oxidative stress and ischemia-reperfusion injury can induce acute cerebral infarction [9] and aggravate the progression of the disease. After acute cerebral infarction, most patients present with limb weakness, sensory disorders, swallowing disorders and cognitive function decline, etc.

Table 3. Logistic regression analysis.

Factor	B	SE	Wald	*p*	OR (95%CI)
Age	0.378	0.071	28.544	<0.001	1.46 (1.27-1.68)
Hypertension	1.391	0.600	5.370	0.020	4.02 (1.24-13.03)
Carotid stenosis	1.503	0.582	6.670	0.010	4.50 (1.44-14.07)
Homocysteine	1.471	0.268	30.079	<0.001	4.35 (2.57-7.37)
Fibrinogen	1.396	0.478	8.551	0.003	4.04 (1.59-10.30)
Constant	-42.356	6.274	45.580	<0.001	-

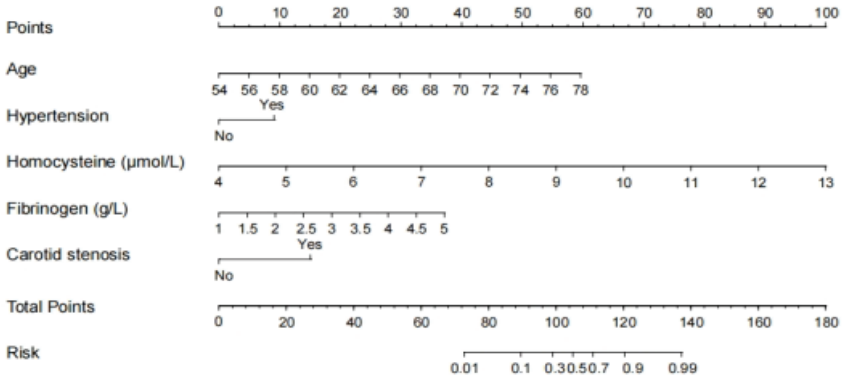


Fig. 1. Nomogram model.

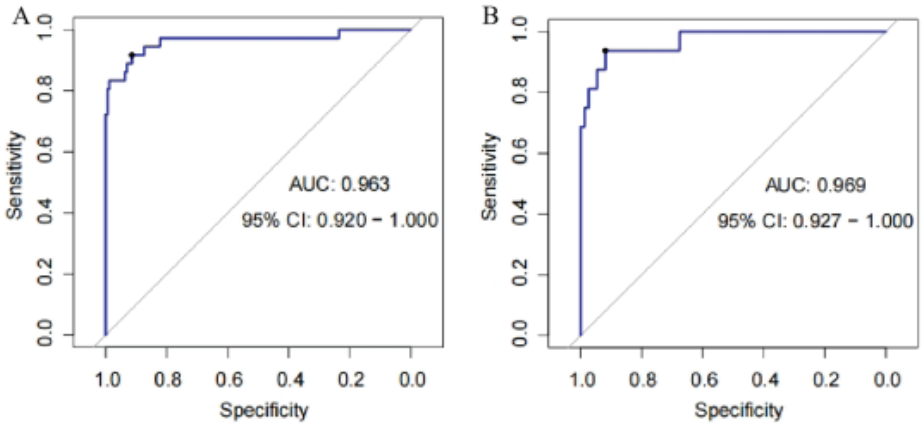
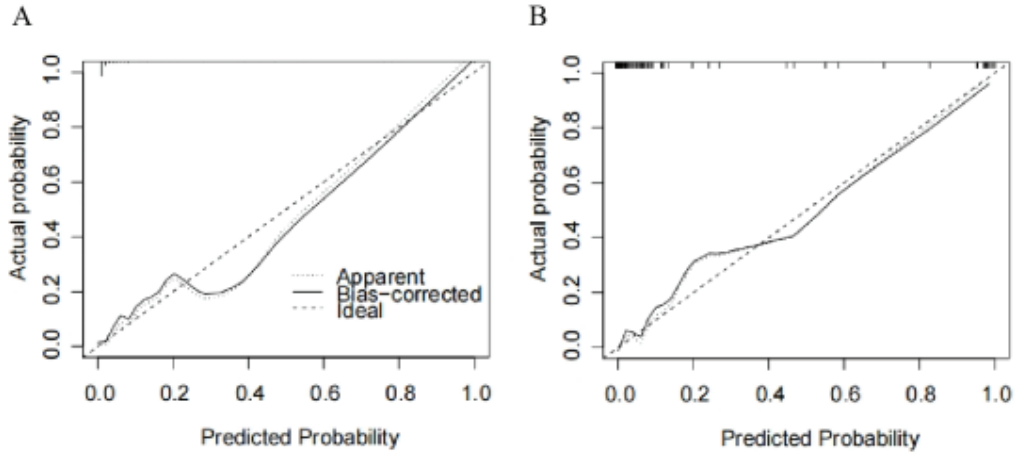


Fig. 2. ROC curve.

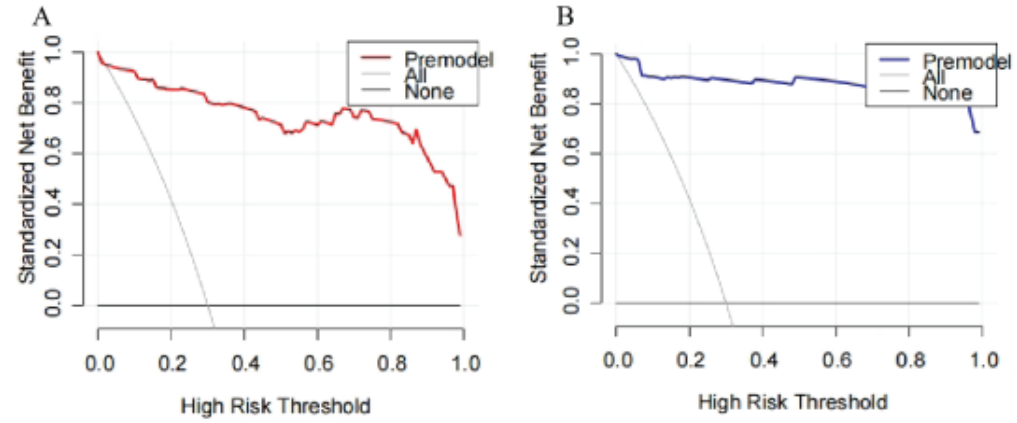
In severe cases, coma or even death may occur. If adequate measures are not taken as soon as possible, a large number of neurons may be damaged [10], thus affecting the neurological function of patients. Intravenous thrombolytic therapy within the time window is an effective means to treat patients with acute cerebral infarction. However, some patients have passed the time window and are not suitable for thrombolytic therapy. Antiplatelet aggregation therapy is a common clinical intervention for acute cerebral infarction [11]. However, its therapeutic effect and prognosis are influenced by numerous factors, and the responses of different patients vary significantly. The results of this study showed that 52 cases (17.33%) of 300 ACI patients who passed the hyperthrombolytic time window had a poor prognosis after dual-antiplatelet therapy, consistent with the report of Liu et al. [12].

The results of this study showed that old age, history of hypertension, elevated homocysteine level, elevated fibrinogen level, and carotid artery stenosis were risk factors for poor prognosis of ACI patients treated with dual antiplatelet therapy.



Note: A: Training set; B: Test set.

Fig. 3. Calibration curve analysis.



Note: A: Training set; B: Test set.

Fig. 4. Decision curve analysis of the nomogram model.

This study found that advanced age was a risk factor for poor prognosis in ACI patients, which was consistent with Zarrintan[13], who pointed out that elderly patients had degraded body function, weakened arterial elasticity, and were more likely to suffer from arterial stenosis and aggravate atherosclerosis. With the increase of age, the structure and function of cerebral vessels undergo significant changes, such as the decrease of the elasticity of blood vessel walls and the aggravation of atherosclerosis [14]. These changes make that in elderly patients after ACI, the brain tissue's tolerance to ischemia and hypoxia decrease, and the ability to recover nerve function weakens. In addition, the ability of cells to repair and regenerate is reduced in older patients, and the plasticity of neurons is diminished, resulting in slower and less effective recovery of neural function compared to younger patients, thereby increasing the risk of a poor prognosis. The history of hypertension is also one of the risk factors affecting the poor prognosis of ACI patients, which is consistent with the results of Zheng's study[15]. Long-term hypertension leads to vascular endothelial damage, platelet adhesion and aggregation, and accelerates the formation of atherosclerotic plaque[16]. In addition, hypertension also promotes the proliferation of vascular smooth muscle cells, which thickens the vascular wall and narrows the lumen, further damaging hemodynamic stability [17]. After a cerebral infarction, narrow and atherosclerotic blood vessels severely impede the supply of blood to ischemic brain tissue. Even if platelet aggregation is inhibited by dual antiplatelet therapy, established vascular lesions and disordered blood flow status still seriously interfere with the restoration of blood supply to the brain, thereby increasing the risk of poor prognosis.

The results of this study showed that increased homocysteine levels was a risk factor for poor prognosis of patients with ACI treated with dual antiplatelet. Relevant studies have shown that the plasma homocysteine level of ACI patients is significantly higher than that of the general population [18]. The possible reason is that homocysteine is a sulphur-containing amino acid, and its elevated level can damage vascular endothelial cells, promote inflammation and oxidative stress, and accelerate the formation of atherosclerotic plaque. In addition, homocysteine can also directly activate coagulation factors, increase blood coagulation and promote the formation of thrombus [19]. During the hyperthrombolytic time window therapy for cerebral infarction, this tendency of hypercoagulation interacts with vascular lesions, hindering the reperfusion process of ischemic brain tissue, aggravating nerve injury, and leading to poor prognosis. Carotid artery stenosis is a risk factor for poor prognosis of ACI patients treated with dual antiplatelet therapy. It has been reported that plasma homocysteine level is positively correlated with the occurrence and severity of carotid artery stenosis [20]. Carotid artery stenosis significantly reduces the blood supply to the brain, leaving the brain tissue in a fragile state of chronic ischemia and hypoxia. During the occurrence of ACI, the blood supply of ischemic penumbra is severely limited due to carotid artery stenosis, which cannot effectively meet the oxygen and nutrients required for the repair of damaged brain tissue, thus expanding the scope of brain tissue injury and increasing the difficulty of nerve function recovery [21]. When carotid artery stenosis and homocysteine level increase, the two cooperate to aggravate vascular disease and blood hypercoagulability. In the course of dual antiplatelet therapy, this combined effect will weaken the therapeutic effect and make brain tissue ischemia and hypoxia continue to worsen, thereby jointly increasing the risk of poor prognosis of patients. Increased fibrinogen level is also a risk factor for poor prognosis of ACI patients treated with dual antiplatelet therapy, which is consistent with the results of the study [22]. The possible reason is that fibrinogen is a key protein in the process of blood coagula-

tion, and its increased level will enhance the coagulation ability of blood and promote the formation and stability of thrombosis. In ACI patients, elevated fibrinogen levels not only increase the burden of cerebral thrombosis and hinder the recovery of cerebral blood flow, but also further damage brain tissue by activating coagulation factors and inflammatory response [23]. Fibrinogen can also bind to platelets, enhancing the aggregation and activation of platelets, weakening the effect of dual-antiplatelet therapy, and making the disease of cerebral infarction difficult to control effectively.

Additionally, this study constructs a nomogram risk model based on the associated factors. The nomogram can integrate additional clinicopathological parameters to achieve more individualized predictions. It is a calculation chart, rather than complex formulas, presenting the results of regression analysis in an intuitive graphical way. The results of this study showed that the AUC values of the area under the ROC curve of the test set and validation data set of the nomogram model were 0.963 (95%CI: 0.920~1.000) and 0.969 (95%CI: 0.920~1.000), respectively, and the AUC values of the two datasets were >0.75, indicating that the nomogram had good differentiation. The results of this study demonstrate that a model for predicting poor prognosis in ACI patients treated beyond the hyperthrombolytic time window has been successfully established. Following validation in other populations, this nomogram can aid clinicians in informed decision-making regarding ACI.

However, this study still has certain limitations. First, this study is a retrospective analysis with a small sample size, and the conclusions may be biased. Second, due to limitations in the data source, we were unable to include all possible influencing factors, which may limit the comprehensiveness of the constructed model. Future studies can further enhance the reliability of the findings by increasing the sample size and incorporating additional potential influencing factors. At the same time, the nomogram model established in this study exhibits a high degree of differentiation and fit, providing a valuable tool for clinicians in treatment decision-making.

In summary, the factors that affect the prognosis of patients with ACI after the hyperthrombolytic time window include advanced age, history of hypertension, elevated homocysteine level, elevated fibrinogen level, and carotid artery stenosis. The nomogram model, established based on multiple influencing factors, exhibits a high degree of differentiation and fit, providing clinicians with tools to optimize treatment and reduce disability and mortality.

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Consent to publish

The manuscript has neither been previously published nor is it under consideration by any other journal. The authors have all approved the content of the paper.

Consent to participate

We secured a signed informed consent form from every participant.

Ethic approval

The Tianjin Hospital Ethics Committee approved this study.

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Conflicts of interest

The authors declare that they have no financial conflicts of interest.

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Author contribution

JC, developed and planned the study, interpreted the results and edited and refined the manuscript with a focus on critical intellectual contributions. YH, participated in collecting, assessing, and interpreting the data and made significant contributions to the data interpretation and manuscript preparation. ML, provided substantial intellectual input during the drafting and revision of the manuscript.

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Novedosos agentes dopaminérgicos y serotoninérgicos derivados del quinolin-metiladamantano y amino-metiladamantano. Síntesis y perfil farmacológico.

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Palabras clave: serotonina; dopamina; enfermedad de parkinson; agentes antipsicóticos; ansiolíticos.

Resumen. La serotonina (5-HT) y la dopamina (DA) son neurotransmisores claves en el sistema nervioso central (SNC) que participan en la regulación del comportamiento. La disfunción de las vías de neurotransmisión dopaminérgica y serotoninérgica, que son responsables de la regulación del estado de ánimo, la cognición y el movimiento, puede provocar una amplia gama de trastornos cerebrales, tanto neurológicos como psiquiátricos. La DA está implicada en diversas enfermedades neurodegenerativas y neuropsiquiátricas y se conoce que los sistemas dopaminérgicos en el cerebro reciben inervación serotoninérgica. En el presente estudio se presenta la síntesis y la evaluación farmacológica preliminar de los compuestos *N*-[(2-cloro-quinolin-3-il)metil]-metiladamantano (**7**) y amino-metiladamantano (**10**), que resultaron tener actividades dopaminérgicas y serotoninérgicas en el sistema nervioso central. La estrategia utilizada en el diseño del compuesto híbrido **7** permitió obtener un novedoso agente con actividad antipsicótica y/o ansiolítica. El compuesto **10** sólo mostró actividad antiparkinsoniana. Estos compuestos constituyen un aporte al arsenal farmacológico para el tratamiento de patologías relacionadas con las enfermedades neurodegenerativas y neuropsiquiátricas.

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Novel dopaminergic and serotoninergic derivative agents from quinoline-methyladamantane and amino-methyladamantane. Synthesis and pharmacological profile.

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Keywords: serotonin; dopamine; parkinson disease; antipsychotic agents; anti-anxiety agents.

Abstract. Serotonin (5-HT) and dopamine (DA) are key neurotransmitters in the central nervous system (CNS) that are involved in the regulation of behavior. Dysfunction of dopaminergic and serotonergic neurotransmitter pathways, which are responsible for the regulation of mood, cognition, and movement, can lead to a wide range of brain disorders, both neurological and psychiatric. DA is implicated in various neurodegenerative and neuropsychiatric diseases, and dopaminergic systems in the brain are known to receive serotonergic innervation. In the present study, we present the synthesis and preliminary pharmacological evaluation of the compounds N-[(2-chloro-quinolin-3-yl) methyl]-methyladamantane (**7**) and amino-methyladamantane (**10**), which were found to have dopaminergic and serotonergic activities in the central nervous system. The strategy used in the design of hybrid compound **7** led to the development of a novel agent with antipsychotic and/or anxiolytic activity. Compound **10** only showed antiparkinsonian activity. These compounds contribute to the pharmacological arsenal for the treatment of pathologies related to neurodegenerative and neuropsychiatric disorders.

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INTRODUCCION

La serotonina (5-HT) y la dopamina (DA) son neurotransmisores claves en el sistema nervioso central (SNC) que participan en la regulación del comportamiento. La disfunción de las vías de neurotransmisión dopaminérgica y serotoninérgica, que son responsables de la regulación del estado de ánimo, la cognición y el movimiento, puede provocar una amplia gama de trastornos cerebrales, tanto neurológicos como psiquiátricos [1]. Así, la DA está implicada en diversas enfermedades neurodegenerativas y neuropsiquiátricas, incluyendo la enfermedad de Parkinson, la disquinesia tardía, el síndrome de Tourette, la enfermedad de Huntington, la esquizofrenia, adicciones, la manía, la depresión y los trastornos de la alimentación [2-7]. La DA no solo es un intermediario en la síntesis de catecolaminas, sino que también, en el SNC, juega un papel crucial en la regulación de funciones como la activación (alerta y vigilia), atención, iniciación de movimientos, percepción, motivación y emociones. Estas funciones se manifiestan a través de diferentes sistemas y vías dopaminérgicas en el cerebro. La dopamina se encuentra en dos formas rotámeras, α y β, y su acción depende de su concentración, ubicación y tiempo de liberación. La acción de la DA esta mediada a través de su interacción con receptores específicos, que se clasifican en cinco subtipos (D_1, D_2, D_3, D_4 y D_5). Desde una perspectiva farmacológica, estos subtipos se agrupan en dos clases principales: los receptores D_1 (D_1

y D_5) y los receptores D_2 (D_2, D_3 y D_4) [8-10]. La DA se encuentra principalmente en la sustancia negra, el cuerpo estriado, el núcleo caudado y el sistema límbico, donde desempeña un papel fundamental en la motricidad, la memoria, el aprendizaje, el estado de ánimo y el sistema de recompensa [11]. Igualmente, la DA está directamente implicada en la enfermedad de Parkinson (EP) y en la enfermedad de Huntington (EH), ambas neurodegenerativas, crónicas y progresivas. La EP se caracteriza por la pérdida de neuronas que producen DA en los ganglios basales, lo que causa problemas de movimiento, mientras que la EH, aunque no está directamente relacionada con la dopamina, también afecta a los ganglios basales y causa trastornos en el movimiento [11-13].

Los sistemas dopaminérgicos en el cerebro reciben inervación serotoninérgica (5-HT) que proviene principalmente del núcleo dorsal del rafe y del núcleo rafe medial. Estas fibras serotoninérgicas modulan la actividad de las neuronas dopaminérgicas en áreas como el área tegmental ventral (ATV), en la sustancia negra (SN), así como también al núcleo accumbens, a la corteza prefrontal y al cuerpo estriado. La acción 5-HT está mediada por siete clases de receptores. Los receptores 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_3 y 5-HT_4 facilitan la liberación de DA, mientras que los 5-HT_{2C} inhiben su liberación. La serotonina y sus receptores (5-HT_{1A}, 5-HT_{1B}, y 5-HT_{2C}) en los ganglios basales juegan un papel importante en el desarrollo de nuevas drogas para la enfermedad de Parkinson, ya que la serotonina puede modular la actividad dopaminérgica, la cual es deficiente en esta enfermedad. La estimulación de los receptores 5-HT_{1A} en los ganglios basales y el cuerpo estriado puede reducir la liberación de 5-HT y disminuir el tono glutamatérgico, lo que podría tener efectos antiparkinsonianos y antidiscinésicos. En el globo pálido y la sustancia negra (SN), la estimulación de los receptores 5-HT_{1B} reduce la liberación de ácido gamma-aminobutírico (GABA), lo que puede tener un efecto antiparkinsoniano y antidiscinésico. Esta reducción de GABA, un neurotransmisor inhibidor, permite que los circuitos motores de los ganglios basales funcionen de manera más efectiva, lo que podría mejorar los síntomas de enfermedades como la enfermedad de Parkinson. Al estimular los receptores 5-HT_{2C} en la sustancia negra reticular (SNr) y el segmento medial del complejo palidal, se puede aumentar la sobreactividad en el Parkinsonismo. Por lo tanto, bloquear estos receptores podría tener un efecto antiparkinsoniano. En el contexto de las discinesias inducidas por la levodopa en la enfermedad de Parkinson, la activación de los receptores 5-HT_{2C} mediante agonistas de la dopamina (como pramipexol o ropinirol) puede tener un efecto antidiscinésico. Estos agonistas imitan la acción de la dopamina, lo que puede ayudar a reducir o disminuir los movimientos involuntarios [14].

Con relación a los trastornos neuropsiquiátricos, hay evidencia que indica que el estrés juega un papel fundamental en la fisiopatología de la depresión y que altera de manera compleja la regulación de la dopamina en el sistema mesolímbico, una región cerebral implicada en el procesamiento de recompensas y emociones. Este sistema se ve afectado por el estrés en términos de liberación, metabolismo y densidad de receptores para DA [15]. Se ha sugerido que el bloqueo de los receptores 5-HT_{2C} puede aumentar la liberación de dopamina y norepinefrina, lo que puede tener efectos antidepresivos y sugiere el bloqueo de los receptores 5-HT_{2C} para ejercer efectos antidepresivos [16]. Varias drogas psicotrópicas producen regulación por disminución de los receptores 5-HT_{2C} y se sugiere que la adaptación de esos receptores tiene un papel en su acción terapéutica. El tratamiento crónico con agonistas o antagonistas de los receptores 5-HT_{2C} se asocia con una regulación a la baja (*downregulation*) o disminución de los receptores 5-HT_{2A} y 5-HT_{2C}. Esto significa que, con el uso prolongado de estos medicamentos, el cuerpo reduce la cantidad o la sensibilidad de estos receptores en respuesta a la exposición

prolongada al agonista o antagonista [17]. Así mismo, los receptores 5-HT_3 podrían desempeñar un papel en la regulación de la liberación de DA y los antidepresivos que bloquean los receptores 5-HT_{2C} y que son agonistas de los 5-HT_3 podrían mejorar los síntomas depresivos más rápidamente. Esta idea se basa en la hipótesis que la 5-HT regula los niveles de DA, y que los receptores 5-HT_3 podrían estar involucrados en esta interacción, especialmente en el núcleo accumbens [14,18].

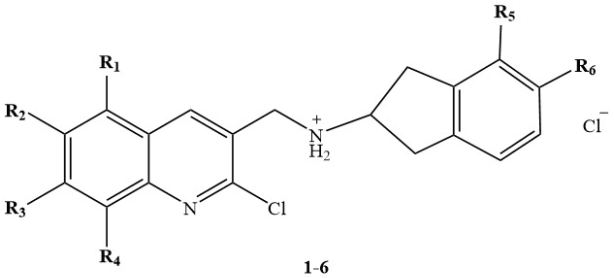
Por su parte se sabe que, en la esquizofrenia, existe una hipersecreción de DA en las vías mesolímbicas, responsables de los síntomas positivos, mientras que, el bloqueo de la respuesta dopaminérgica por acción de los antipsicóticos en las proyecciones corticales se relaciona con los síntomas negativos y los trastornos cognitivos. Los antipsicóticos típicos, también conocidos como de primera generación, ejercen sus efectos clínicos al bloquear los receptores D_2 en la vía mesolímbica, lo que reduce los síntomas positivos de la esquizofrenia, como alucinaciones y delirios. Al mismo tiempo, el bloqueo de los receptores D_2 en la vía nigroestriatal causa efectos extrapiramidales, como temblores, rigidez y movimientos anormales [19]. Ahora bien, el mecanismo responsable de los efectos clínicos de los antipsicóticos atípicos no es totalmente conocido y diversos estudios indican que estimulan la liberación de DA más potentemente en la corteza frontal medial y en las áreas mesolímbicas que en el cuerpo estriado. Esta acción selectiva se asocia con baja incidencia de efectos adversos extrapiramidales y con mayor eficacia para mejorar los síntomas negativos y cognitivos de la esquizofrenia. Una propiedad común de este grupo de drogas es su elevada afinidad por los receptores 5-HT_{2A} y se ha sugerido que el antagonismo sobre estos receptores, en conjunto con una acción antagonista débil sobre los receptores D_2, sería responsable de los efectos clínicos beneficiosos [20]. Por otro lado la acción facilitadora ejercida por la estimulación de los receptores 5-HT_{1A} aumenta la liberación de DA a nivel cortical. La combinación de la acción de un antagonista o agonista parcial sobre los receptores D_2 y la acción agonística sobre los receptores 5-HT_{1A} es una de las nuevas terapias para desarrollar en la búsqueda de nuevos antipsicóticos [21]. Los antipsicóticos atípicos probablemente aumentan la liberación de DA en la corteza prefrontal por bloqueo de los receptores 5-HT_{2C}, y reducen los efectos extrapiramidales y además mejoran los síntomas negativos de la esquizofrenia[22]. Por otro lado, el bloqueo de la neurotransmisión DA en el núcleo accumbens es el mecanismo principal que subyace a la mejoría de los síntomas positivos. Entonces, una estrategia posible sería reducir la actividad de la vía mesolímbica por bloqueo de los receptores D_2 sin afectar la actividad de la vía nigroestriada. Los efectos selectivos de los antagonistas 5-HT_{2C} sobre la vía mesolímbica sugieren que podrían tener eficacia antipsicótica sin producir efectos adversos extrapiramidales. Diversos datos recientes indican que la combinación de bloqueo D_2 y el antagonismo sobre los recetores 5-HT_{2A} y 5-HT_{2C} sería el mecanismo de acción de los nuevos antipsicóticos [14].

La investigación y el desarrollo de fármacos que actúen como agonistas parciales en los receptores D_2 y 5-HT_{1A} ha sido considerado para el tratamiento de la esquizofrenia, la depresión mayor, el trastorno bipolar y la enfermedad de Parkinson. Estos fármacos, como el aripiprazol y el brexpiprazol, son ejemplos de antipsicóticos de tercera generación que se utilizan para tratar la esquizofrenia y otros trastornos. Los agonistas parciales se comportan como moduladores, ya que su eficacia o actividad intrínseca dependen del tipo de receptor y de las concentraciones del neurotransmisor natural; así que estas drogas, podrían restaurar la neurotransmisión adecuada mientras que induce menos efectos secundarios [23].

Desde el punto de vista Químico Medicinal la búsqueda de nuevos compuestos capaces de contrarrestar las enfermedades neuropsiquiátricas y neurodegenerativas, ha sido de gran importancia. Para ello, Angel y col., [24-29] diseñaron, sintetizaron y evaluaron

farmacológicamente los análogos del clorhidrato de N-2-cloro-(mono o di-metilquinolin-3-il-metil)-2-aminoindano (**1-6**) (Fig. 1), con el fin de obtener nóveles agentes que podrían ser capaces de aliviar algunas de estas patologías, tales como la enfermedad de Parkinson, la esquizofrenia y la Corea de Huntington. Estos compuestos mostraron actividad dopaminérgica central y actividad anti-Corea de Huntington. De aquí se desprende que el fragmento quinolin-metilamino, guarda las aproximaciones básicas como un nuevo farmacóforo dopaminérgico.

En el presente trabajo se presenta la síntesis y la evaluación farmacológica de los compuestos *N*-[(2-cloro-quinolin-3-il)metil]-metiladamantano (**7**) y amino-metiladamantano (**10**) bajo sus sales clorhídricas, ello con el propósito de obtener compuestos con posible actividad moduladora de la transmisión dopaminérgica central que contribuyan con el arsenal farmacológico para el tratamiento de patologías relacionadas con las enfermedades neurodegenerativas y neuropsiquiátricas.



1-6

Compuesto	R_1	R_2	R_3	R_4	R_5	R_6
1	H	H	H	H	H	H
2	H	Me	H	H	H	H
3	H	H	Me	H	H	H
4	H	H	H	Me	H	H
5	Me	H	H	Me	H	H
6	H	H	H	H	OMe	OMe

Fig. 1. Sustituyentes de los compuestos (**1-6**).

MATERIALES Y MÉTODOS

SECCIÓN QUÍMICA

Los compuestos (**7**) y (**10**) fueron obtenidos de acuerdo a la ruta sintética planteada (Fig. 2), y su presencia se corroboró de acuerdo a sus datos espectroscópicos de RMN ^{1}H y ^{13}C, punto de fusión y análisis elemental [24-30]. Todos los reactivos y solventes utilizados en la síntesis fueron de grado analítico, en los casos necesarios, los solventes se sometieron a procesos previos de secado mediante métodos estándar. Los espectros de resonancia magnética nuclear de ^{1}H y ^{13}C se realizaron en un espectrómetro Jeol Eclipse 270 (270 MHz/67.9 MHz) ubicado en la Facultad de Farmacia-UCV. Los desplazamientos químicos son reportados en partes por millón (ppm). Los puntos de fusión se determinaron en un aparato Stuart Scientific y están sin corregir. Los procesos de hidrogenación catalítica se realizaron utilizando un aparato Parr de baja presión, empleando para ello Pd/C al 10% como catalizador. El análisis elemental fue realizado en un equipo Perkin Elmer 2400 CHN Elemental Analyzer, los resultados están en el rango de ± 0,4% del su valor teórico.

Síntesis de 2-cloro-3-formilquinolina 12

Fueron añadidos en un balón de 3 bocas, 4 mL (51,66 mmol) de N, N-dimetilformamida con agitación constante, y colocado en un baño de hielo. Luego de esto se agregaron, gota a gota, 7 mL (75,09 mmol) de cloruro de fosforilo ($POCl_3$) con la ayuda de un embudo de adición, manteniendo temperatura menor a 20°C, para la formación del reactivo de Vilsmeier, posteriormente fue retirado el embudo y adicionados 1 g (5,23 mmol) de acetanilida (**11**) en 2 partes; la mezcla resultante fue sometida durante 4 horas a reflujo manteniendo temperatura constante en 100°C. Finalizado el tiempo de reacción fue agregado hielo cuidadosamente y el sólido resultante de color amarillo fue filtrado, lavado y secado; obteniéndose 0,61 g del producto cuyo punto de fusión fue de 149-150°C (Lit[25]. p.f. 149°C), con un rendimiento de 61%.

Síntesis del 1-nitro-metiladamantano 9

Se colocaron 100 mL de dimetilsulfóxido (DMSO) seco en un balón de 3 bocas bajo atmósfera de nitrógeno y con agitación constante. Posteriormente fueron añadidos pro-

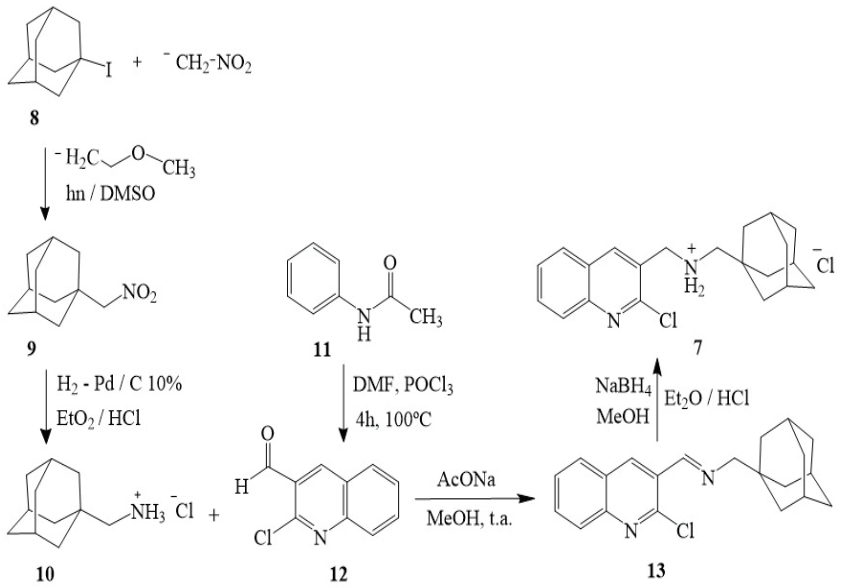


Fig. 2. Ruta para la síntesis de los compuestos **7** y **10**.

panona (2 mmol) y nitrometano (3 mmol), al transcurrir 15 minutos se agregó el compuesto **8** (1 mmol). La mezcla fue irradiada en un reactor con dos lámparas UV de 400W por un período de 6 horas; al finalizar este tiempo la reacción fue detenida incorporando un exceso de nitrato de amonio. El producto resultante fue disuelto en 300 mL de agua y extraído con 100mL de éter etílico y el residuo fue purificado por cromatografía de columna en sílica gel, usando como eluyente una mezcla de éter de petróleo – éter etílico (98:2) obteniendo un aceite amarillo con un 27% de rendimiento.

RMN ^{1}H (δ, ppm): 1.70 – 2.50 (m, 15H, Ad), 4.30 (s, 2H, C$\underline{H}_2$).

Síntesis del Clorhidrato de Amino-metiladamantano 10

En un recipiente de vidrio para hidrogenar se colocaron 0,43 g (2,20 mmol) del compuesto **9** este fue disuelto con 1,2 mL de ácido clorhídrico (HCl) concentrado y 20 mL de etanol (EtOH) absoluto a temperatura ambiente, para luego ser agregados cuidadosamente 0,2 g de Pd-C 10%, la mezcla fue sometida a una presión de 10 psi hasta consumirse la cantidad calculada de hidrógeno. Posteriormente el catalizador fue removido por filtración y en el filtrado resultante se eliminó el solvente a presión reducida, obteniendo un aceite amarillo que al ser tratado con una mezcla éter-HCl se observó la formación de 0,25 g de un sólido color crema de punto de fusión 298°C, con un rendimiento de 58,14%.[28-29]

Anal Teórico: C, 65.49; H. 9.99; N, 6.94. Encontrado: C, 65.53; H. 10.01; N, 7.23.

RMN ^{1}H($CDCl_3$) (δ, ppm): 1.57 – 2.01 (m, 15H, Ad), 2.64 (s, 2H, C$\underline{H}_2$)

RMN ^{13}C($CDCl_3$) (δ, ppm): 27.82, 32.11, 36.25, 39.55 (C$\underline{H}$ y C$\underline{H}_2$ Ad), 51.11 (C$\underline{H}_2$-Ad).

Síntesis de Clorhidrato N-[(2-cloro-quinolin-3-il) metil]-metiladamantano 7

En un balón de 100 mL se añadieron 0,12 g (0,60 mmol) del compuesto **10**, 0,095 g (0,5 mmol) del compuesto **12** y 0,05 g (0,61 mmol) de acetato de sodio, empleando 8 mL de metanol (MeOH) como solvente. La mezcla resultante fue colocada en agitación constante a temperatura ambiente, hasta la aparición de un precipitado que fue filtrado por gravedad, lavado con metanol (MeOH) y secado en la estufa a presión reducida a 60°C. Seguidamente en un balón de 100 mL, se pesaron 0,55 g (0,16 mmol) del sólido blanco (la imina **13)** y se agregaron 6 mL

de metanol (MeOH) y $NaBH_4$ (0,01 g, 0,159 mmol). Esta mezcla fue colocada en un baño de hielo con agitación constante por 24 horas y una vez concluido este lapso, se añadió 1 mL de ácido clorhídrico (HCl) concentrado y 20 mL de agua; el solvente fue evaporado a presión reducida y la solución acuosa resultante se alcalinizó hasta llevarla a pH 10 empleando granallas de hidróxido de sodio (NaOH). La extracción se realizó con diclorometano (CH_2Cl_2) y los extractos orgánicos combinados, se lavaron con agua destilada y se secaron con sulfato de sodio anhidro, se filtró por gravedad y el solvente fue evaporado a presión reducida. El aceite resultante se trató tratado con una mezcla éter-HCl, obteniéndose un sólido blanco amorfo, recristalizado en isopropanol-éter para dar 0,036 g de un sólido blanco (52,94%) con un punto de fusión de 222°C.[26]

Anal Teórico: C, 66.84; H. 6.95; N, 7.42. Encontrado: C, 66.89; H. 7.03; N, 7.31.

^{1}H-RMN (MeOH-d_3) **δ:** 1,72 ppm (m, 12H, 6C<u>H</u>$_2$ Ad); 2.04 ppm (s, 3H, 3C<u>H</u> Ad); 2,95 ppm (s, 2H, C<u>H</u>$_2$ Ad (C_{10})); 4,58 ppm (s, 2H, C<u>H</u>$_2$ Quinolina (C_9)); 7,72 ppm (psdt, 1H, C<u>H</u>, Ar-<u>H</u> quinolina (C_6) J= 6,9 Hz y J= 1,2 Hz); 7,89 ppm (psdt, 1H, C<u>H</u>, Ar-H quinolina (C_7), J= 7,7 Hz, J= 6,9 Hz y J= 1,2 Hz); 8,0 ppm (d, 1H, C<u>H</u>, Ar-<u>H</u> quinolina (C_5) J= 8,39 Hz); 8,06 ppm (d, 1H, C<u>H</u>, Ar-<u>H</u> quinolina (C_8) J= 8,39 Hz); 8,69 ppm (s, 1H, C<u>H</u>, Het-quinolina (C_4)).

^{13}C-RMN (MeOH-d_3) **δ:** 28,10 ppm (3<u>C</u>H Ad); 36,08 y 39,25 ppm (6<u>C</u>H$_2$ Ad); 49,70 ppm (<u>C</u>H$_2$ (C_9-quinolina)); 60,05 ppm (<u>C</u>H$_2$ (C_{10}-Adamantano)); 123,28 ppm, 126,96 ppm, 127,54 ppm, 127,96 ppm, 128,09 ppm, 131,91 ppm, 141,83 ppm, 147,70 ppm, 150,02 ppm (<u>C</u>H, aromáticos quinolina).

RMN-HETCOR (MeOH-d_3) **δ:**28,10 ppm (3<u>C</u>H Ad) se correlaciona con la señal a 2.04 ppm (s, 3H, 3C<u>H</u> Ad); a 36,08 y 39,25 ppm (6<u>C</u>H$_2$ Ad) se correlaciona con la señal a 1,72 ppm (m, 12H, 6C<u>H</u>$_2$ Ad); a 49,70 ppm (<u>C</u>H$_2$ (C_9-quinolina)) se correlaciona con la señal a 4,58 ppm (s, 2H, C<u>H</u>$_2$ Quinolina (C_9)); a 60,05 ppm (<u>C</u>H$_2$ (C_{10}-Adamantano)) se correlaciona con la señal a 2,95 ppm (s, 2H, C<u>H</u>$_2$ Ad (C_{10})); a 127,52 ppm (<u>C</u>H, aromático (C_5)) se correlaciona con la señal a 8,0 ppm (d, 1H, C<u>H</u>, Ar-<u>H</u> quinolina (C_5) J= 8,39 Hz) a 127,97 ppm (<u>C</u>H, aromático (C_6)) se correlaciona con la señal 7,72 ppm (psdt, 1H, C<u>H</u>, Ar-<u>H</u> quinolina (C_6) J= 6,9 Hz y J= 1,2 Hz); a 128,10 ppm (<u>C</u>H, aromático (C_8)) se correlaciona con la señal a 8,06 ppm (d, 1H, C<u>H</u>, Ar-<u>H</u> quinolina (C_8) J= 8,39 Hz); a 131,92 ppm (<u>C</u>H, aromático (C_7)) se correlaciona con la señal a 7,89 ppm (psdt, 1H, C<u>H</u>, Ar-H quinolina (C_7), J= 7,7 Hz, J= 6,9 Hz y J= 1,2 Hz); a 141,81 ppm (<u>C</u>H, Het-quinolina (C_4)) se correlaciona con la señal a 8,69 ppm (s, 1H, C<u>H</u>, Het-quinolina (C_4)).

SECCIÓN FARMACOLÓGICA

Se utilizaron ratas macho de la cepa *Sprague-Dawley* de 150 a 250 g de peso corporal, mantenidas bajo períodos alternativos de luz y oscuridad, con libre acceso al agua y alimento estándar (Ratarina®, Protinal). Cinco días antes del experimento se les implantó a las ratas, bajo anestesia con cilazina (Setton® al 2%) (1mg/Kg.; i.p) y relajación con ketamina, una cánula metálica en el ventrículo lateral-derecho, según las coordenadas: antero-posterior –0,40 mm del Bregma, 1,2 mm lateral, y 3 mm ventral[26, 28-30]. Las cánulas, empleadas como guía para la introducción de la aguja de inyección intracerebroventricular (ICV), se fabricaron utilizando inyectadoras 20G con un largo inferior a 4 mm, selladas con silicona y fijadas al cráneo permanentemente con cemento acrílico. La inyección ICV se realizó utilizando una inyectadora Hamilton de 10μL provista de un tope para aplicación precisa de los compuestos.

Protocolo para la evaluación de la conducta estereotipada

La conducta estereotipada, definida como una actividad motora repetitiva y sin propósito, se evaluó mediante observación, para lo cual se colocó cada animal dentro de una caja de acrílico transparente con las siguientes dimensiones: 32x28x28 cm, con

un período previo de 15 minutos de habituación. Las observaciones se realizaron durante 60 minutos, divididos en 10 intervalos de 6 minutos cada uno. Para cada una de las pruebas, se utilizaron grupos de cuatro animales y las conductas evaluadas fueron: lamidas, roídas, olfateos y acicalamientos.

Los datos recolectados se registraron empleando una computadora dotada de un software para contar el número de movimientos estereotipados.

Protocolo de administración de los compuestos evaluados

Los compuestos **7** y **10** fueron disueltos en solución salina y se administró vía ICV en un volumen de inyección de 5μL, a las dosis de 50μg/5μL y 5μg/5μL. Como control se utilizó solución salina (5μL). Se emplearon cuatro animales por cada grupo experimental. Todos los pretratamientos fueron realizados por vía intraperitoneal, 15 minutos antes de la administración ICV de los compuestos (**7** y **10**) de la solución salina, y fueron los siguientes: 1. Haloperidol (1mg/Kg) (Haldol 50mg/mL, solución inyectable, Janssen Pharmaceuticals, Inc), antagonista de los receptores dopaminérgicos; 2. Ziprasidona (1mg/ Kg) (Geodon R, Laboratorios Pfizer), antipsicótico atípico; 3. Buspirona (1mg/ Kg) (Clorhidrato de buspirona, Buspar, comprimidos de 20 mg, Dalpas®, Biotech Laboratorios), un agonista parcial de los receptores de serotonina ($5HT_{1a}$) y antagonista de los receptores D_2; 4. Sertralina (dosis de 1 mg/Kg) inhibidor selectivo de la recaptación de serotonina; 5. Fluoxetina (dosis de 1mg/ Kg) inhibidor selectivo de la recaptación de serotonina y antagonista de los receptores ($5HT_{2c}$); 6. Apomorfina (1mg/Kg) (APO-go PEN 10 mg/mL, solución inyectable), agonista dopaminérgico D_1-D_2.

Protocolo de la denervación dopaminérgica central

A un grupo de ratas canuladas ICV se le realizó una denervación química con 6-hidroxidopamina (6-OHDA) (Sigma Aldrich, Saint Louis, MO) (112 mM, pH=7,4). Para ello se inyectó la solución neurotóxica en el ventrículo lateral derecho a una dosis de 200 μg/5μL, 48 y 72 h previas a la administración-ICV de los compuestos **7** y **10** (50μg/5μL, n=4). Como control se usó solución salina (5μL, n=4).

Análisis estadístico

Los resultados fueron expresados como la media ± E.E.M. Los datos fueron evaluados mediante el análisis de varianza de una y dos vías (ANOVA) y la prueba de Newman-Keuls [31]. Un valor de $p<0,05$ fue considerado estadísticamente significativo. El análisis de los resultados y la elaboración de los gráficos se realizaron empleando el programa GraphPad Prism versión 5.1.

RESULTADOS Y DISCUSIÓN

Efecto de los compuestos 7 y 10 administrados por vía ICV sobre la conducta estereotipada en ratas. Efecto del pretratamiento con haloperidol, ziprasidona, buspirona, sertalina, fluoxetina y apomorfina.

La posible participación de los compuestos **7** y **10** sobre los sistemas dopaminérgico y serotoninérgico central están avalados por los resultados farmacológicos que se presentan en las Figs. 3 a la 6, cuando se administraron vía ICV, a las dosis de 50μg/5μL y 5μg/5μL, respectivamente.

Para poder interpretar estos resultados, es necesario conocer el mecanismo de acción de los fármacos utilizados como patrones. La apomorfina es un agonista dopaminérgico que actúa sobre los receptores D_1 y D_2, mientras que el haloperidol es un antagonista de estos mismos receptores y es un antipsicótico típico. En cuanto a la ziprasidona, cuya acción farmacológica se diferencia del haloperidol, por ser un antipsicótico atípico, bloquea los receptores $5HT_{2a}$ y D_2 (en una proporción de 8:1, respectivamente) y es un agonista parcial del receptor $5HT_{1a}$.[32-36]. La buspirona es un agonista parcial postsináptico del receptor $5HT_{1a}$ y

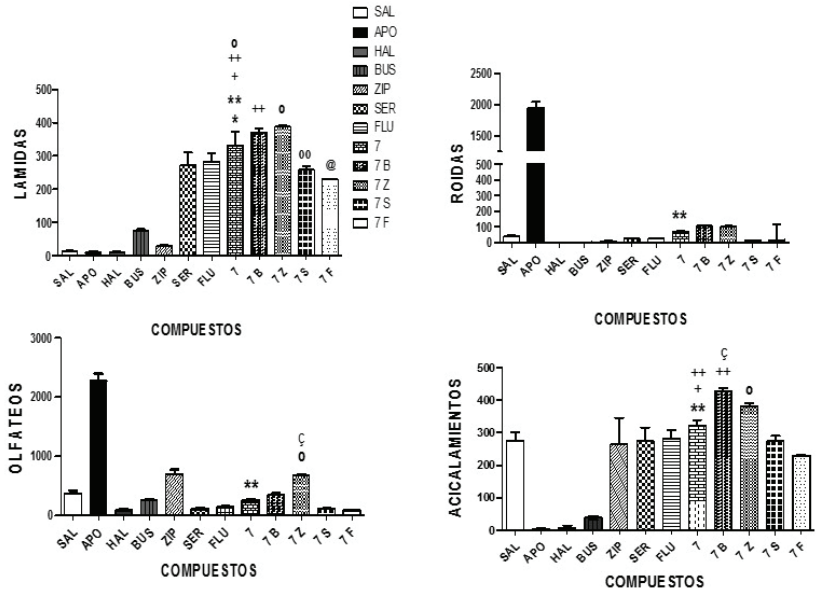


Fig. 3. Efecto del compuesto 7 (5 μg) sobre la conducta estereotipada en ratas. En las ordenadas, la suma de las conductas medidas. En las abscisas, los compuestos evaluados. Las observaciones se realizaron por una hora. Los resultados se expresaron como promedio ± E.E.M. de cuatro mediciones independientes. Los datos fueron analizados mediante análisis de variancia (ANOVA) de una vía y la prueba de Newman-Keuls. *Diferencia Significativa (DS) *vs.* salina (SAL); **DS vs. apomorfina (APO); +DS vs. haloperidol (HAL); ++DS vs. buspirona (BUS); ^{o}DS vs. ziprasidona (ZIP); ^{oo}DS vs. sertralina (SER), @DS vs. fluoxetina (FLU); ÇDS vs. compuesto 7 (5μg). p≤ 0,05.

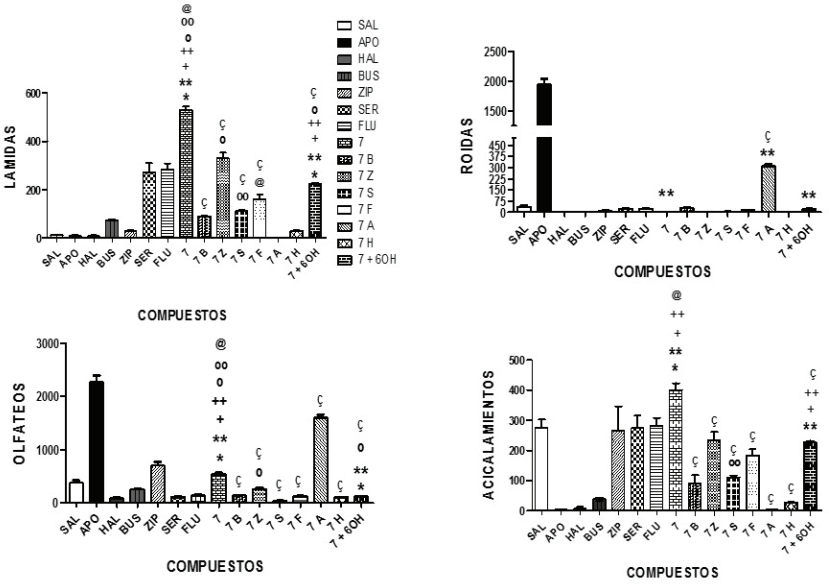


Fig. 4. Efecto del compuesto 7 (50 μg) sobre la conducta estereotipada en ratas. En las ordenadas, la suma de las conductas medidas. En las abscisas, los compuestos evaluados. Las observaciones se realizaron por una hora. Los resultados se expresaron como promedio ± E.E.M. de cuatro mediciones independientes. Los datos fueron analizados mediante análisis de variancia (ANOVA) de una vía y la prueba de Newman-Keuls. *Diferencia Significativa (DS) vs salina (SAL); **DS vs. apomorfina (APO); +DS vs. haloperidol (HAL); ++DS vs. buspirona (BUS); ^{o}DS vs. ziprasidona (ZIP); ^{oo}DS vs. sertralina (SER), @ DS vs. fluoxetina (FLU); Ç DS vs. compuesto 7 (50μg). p≤ 0,05.

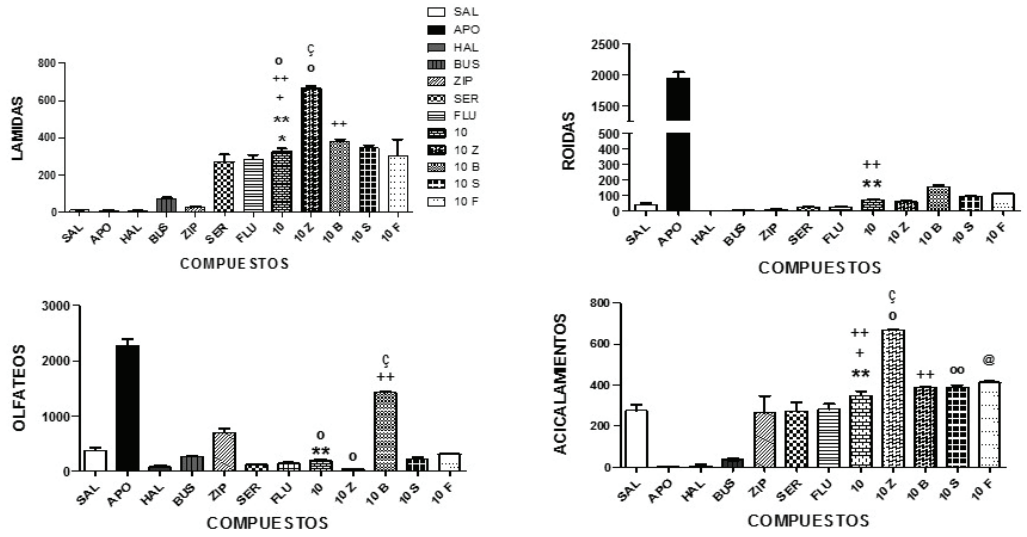


Fig. 5. Efecto del compuesto **10** (5 μg) sobre la conducta estereotipada en ratas. En las ordenadas, la suma de las conductas medidas. En las abscisas, los compuestos evaluados. Las observaciones se realizaron por una hora. Los resultados se expresaron como promedio ± E.E.M. de cuatro mediciones independientes. Los datos fueron analizados mediante análisis de variancia (ANOVA) de una vía y la prueba de Newman-Keuls. *Diferencia Significativa (DS) vs. salina (SAL); ** DS vs. apomorfina (APO); + DS vs. haloperidol (HAL); ++ DS vs. buspirona (BUS); º DS vs. ziprasidona (ZIP); ºº DS vs. sertralina (SER), @ DS vs. fluoxetina (FLU); Ç DS vs. compuesto **10** (5μg). p≤ 0,05.

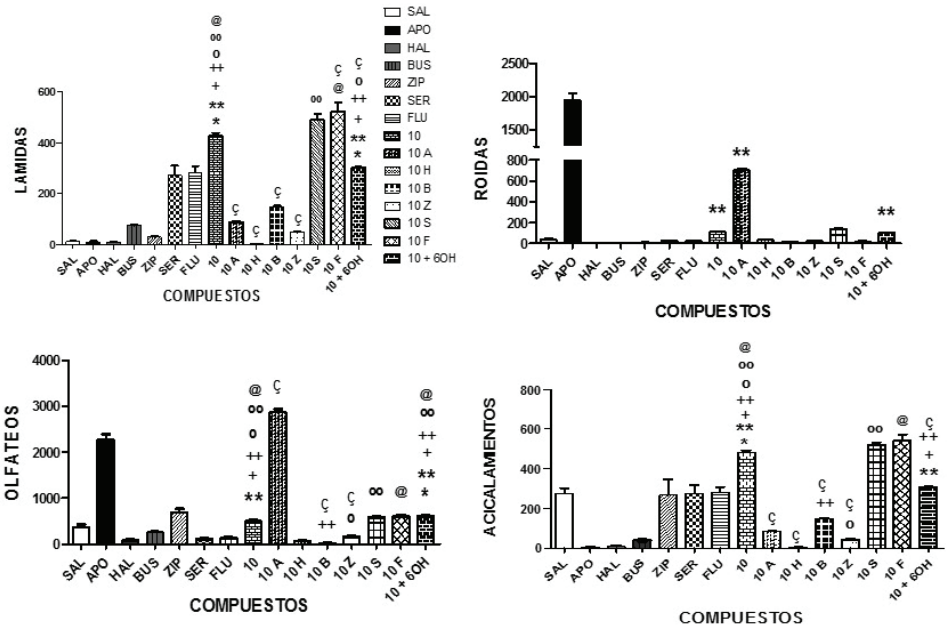


Fig. 6. Efecto del compuesto **10** (50 μg) sobre la conducta estereotipada en ratas. En las ordenadas, la suma de las conductas medidas. En las abscisas, los compuestos evaluados. Las observaciones se realizaron por una hora. Los resultados se expresaron como promedio ± E.E.M. de cuatro mediciones independientes. Los datos fueron analizados mediante análisis de variancia (ANOVA) de una vía y la prueba de Newman-Keuls. *Diferencia Significativa (DS) vs salina (SAL); ** DS vs apomorfina (APO); + DS vs. haloperidol (HAL); ++ DS vs. buspirona (BUS); º DS vs. ziprasidona (ZIP); ºº DS vs. sertralina (SER), @ DS vs. fluoxetina (FLU); Ç DS vs. compuesto **10** (50μg). p≤ 0,05**.**

antagonista débil D_2.[37] Tanto la fluoxetina como la sertralina son antidepresivos que funcionan como inhibidores selectivos de la recaptación de serotonina. Sin embargo, la fluoxetina tiene un mecanismo de acción adicional, también actúa como antagonista del receptor 5-HT_{2c}.[38,39]

Es bien conocido que la estereotipia es el principal componente de varios desórdenes psiquiátricos, incluidos el autismo infantil[34] y la esquizofrenia. Así mismo la conducta de olfateos y roídas, es un comportamiento dependiente de la dopamina y el sustrato neural del comportamiento estereotipado inducido por la apomorfina en animales, se debe a las proyecciones dopaminérgicas de la región de los núcleos caudado y putamen. La apomorfina es un agonista mixto de los receptores de dopamina D_1-D_2. La activación de los receptores de dopamina D_1-D_2 sobre el núcleo estriado se expresa como la respuesta de un comportamiento excesivo y repetitivo (estereotipia)[35-40]. Es decir, que la activación de los receptores dopaminérgicos a nivel del sistema límbico expresa la conducta estereotipada de lamidas y acicalamientos, mientras que los olfateos y roídas son respuestas a la activación de los receptores a nivel del sistema extrapiramidal.

En el presente estudio se observó que los compuestos **7** y **10** por sí solos, indujeron cambios significativos en las respuestas estereotipadas lamidas, acicalamientos, roídas y olfateos a las dosis de 50μg/5μL (Figs. 4 y 6), mientras que a la dosis de 5μg/5μL aumentaron las conductas lamidas, roídas y acicalamientos (Figs. 3 y 5). Las conductas estereotipadas a la dosis de 50μg/5μL, en ambos compuestos, fueron bloqueadas por el antipsicótico típico, haloperidol, demostrando que la actividad de ambos compuestos esta mediada a través de mecanismos dopaminérgicos centrales (Figs. 4 y 6). En las ratas pretratadas con el antipsicótico atípico ziprasidona y el antidepresivo buspirona, la administración ICV de ambos compuestos, a las dosis de 5μg/5μL y 50μg/5μL, produjo cambios significativos de las cuatro conductas estereotipadas, al mostrar un incremento a bajas dosis y un bloqueo altas dosis (Figs. 3 y 6). Estos resultados encontrados están en concordancia con este comportamiento, y corrobora la acción agonística de estos compuestos sobre el receptor $5HT_{1a}$. Efectivamente, la buspirona es un agonista parcial del receptor $5HT_{1a}$ postsináptico y la ziprasidona además de tener actividad sobre los receptores $5HT_{2a}$ y D_2 como antagonista, también actúa como un agonista parcial del receptor $5HT_{1a}$. De manera que la respuesta farmacológica resultante entre un agonista parcial con uno total es un antagonismo a alta dosis y un agonismo a baja dosis.

La denervación presináptica con la neurotoxina 6-OHDA (ICV), inhibió completamente los aumentos marcados en las conductas estereotipadas evaluadas inducidas por los compuestos **7** y **10** a la dosis de 50μg/5μL, lo que sugiere que la acción agonista dopaminérgica del compuesto **7** y **10** no proviene de su acción postsináptica, sino más bien presináptica (Figs. 4 y 6). La acción de los compuestos evaluados podría involucrar entonces receptores en los terminales nerviosos presinápticos, produciendo un incremento del eflujo y/o la depleción del neurotransmisor, o bien por un antagonismo sobre algún receptor dopaminérgico presináptico; mientras que una acción postsináptica estaría interactuando sobre su propio receptor. Aun cuando los resultados demuestran que la acción agonista ejercida por el compuesto **7** y **10** están mediados a través de mecanismos dopaminérgicos centrales, estos parecen no provenir de su acción postsináptica, ya que no hubo aumentos marcados en las conductas estereotipadas evaluadas (Figs. 4 y 6). Sin embargo, aun cuando los resultados sugieren que los compuestos actúan a nivel presináptico, esta aseveración no resulta totalmente concluyente, ya que la respuesta agonística dopaminérgica de ambos compuestos podría resultar de una acción indirecta al depletar el neurotramisor (DA) y/o por la estimulación sobre los receptores $5HT_{1a}$ postsináptico.

Para evaluar la actividad serotoninérgica de estos compuestos, se realizaron pretratamientos con fluoxetina y sertralina. El pretratamiento con sertralina y fluoxetina bloquearon las cuatro conductas estereotipadas inducidas por el compuesto **7**, mientras que produjeron en el compuesto **10** a la dosis de 5μg/5μL un aumentó leve de las lamidas y los acicalamientos, y a la dosis de 50μg/5μL el aumento fue superior, más en las lamidas que en los acicalamientos. El pretratamiento con la apomorfina a la dosis de 50μg/5μL, bloqueó las cuatro conductas estereotipadas de los compuestos **7** y **10**.

Los agonistas del receptor $5HT_{1a}$, aumentan la liberación de dopamina a nivel central. Los antidepresivos fluoxetina y sertralina son inhibidores selectivos de la recaptación de serotonina (5HT) y adicionalmente la fluoxetina es antagonista del receptor $5HT_{2c}$ [36-38]. Es bien conocido que los inhibidores de la recaptación de serotonina conllevan de forma aguda, al aumento de la disponibilidad sináptica de serotonina y por ende la activación de sus receptores postsinápticos. En vista que los compuestos **7** y **10** mostraron actividad agonística sobre el receptor $5HT_{1a}$, se esperaba un aumento significativo de las conductas estereotipadas, cuando se realizó la evaluación con las ratas pretratadas con sertralina y fluoxetina. Sin embargo, el compuesto **7** en ambos pretratamientos bloqueó las lamidas y los acicalamientos en ambas dosis estudiadas mientras que, el compuesto **10** a la dosis de 50μg/5μL sólo aumentó los acicalamientos y las lamidas. Es bien conocido que la fluoxetina también es un antagonista del receptor $5HT_{2c}$ y por ello su respuesta, es aumentar el tono dopaminérgico central [36,37]. El hecho que, las respuestas ejercidas por el compuesto **7** en los animales pretratados con sertralina y fluoxetina hayan sido bloqueadas, es indicativo de su participación agonística sobre el receptor $5HT_{2c}$. Este resultado fue confirmado por la respuesta antagónica ejercida por el compuesto **7** en las ratas pretratadas con apomorfina, al bloquear las conductas roídas y olfateos; mientras que en el compuesto **10** se observa un leve bloqueo de la conducta olfateo en las ratas pretratadas con apomorfina, frente al incremento significativo de esta conducta mostrado por el compuesto **10**. La apomorfina es un agonista de los receptores de dopamina D_1-D_2 y aumenta las conductas roídas y olfateos significativamente, y no así las lamidas y los acicalamientos.[39,40] Pues la actividad de los compuestos **7** y **10** en las ratas pretratadas con apomorfina se debe a la activación del receptor $5HT_{2c}$ (bloqueo significativo de las conductas de los ganglios basales) para el primer compuesto y la acción de agonística sobre los receptores $5HT_{1a}$ para el segundo. Así mismo no se descarta la acción indirecta de estos compuestos sobre las neuronas dopaminérgicas presinápticas.

De acuerdo con los resultados farmacológicos podemos presentar una aproximación químico medicinal sobre el diseño de los compuestos **7** y **10**. El compuesto **7** tiene presente dos fragmentos (quinolin-metil y adamantil-metil) unidos a través de un enlace covalente al grupo amino central y fue diseñado como un compuesto híbrido con dos fragmentos farmacofóricos diferentes. En cuanto al núcleo adamantano, está presente en varios compuestos con actividad farmacológica, tal es el caso que la amantadina (**14**) que aumentan la liberación de dopamina endógena (vía indirecta) y son inhibidores de la recaptación presináptica de dopamina [41-43] (Fig. 7).

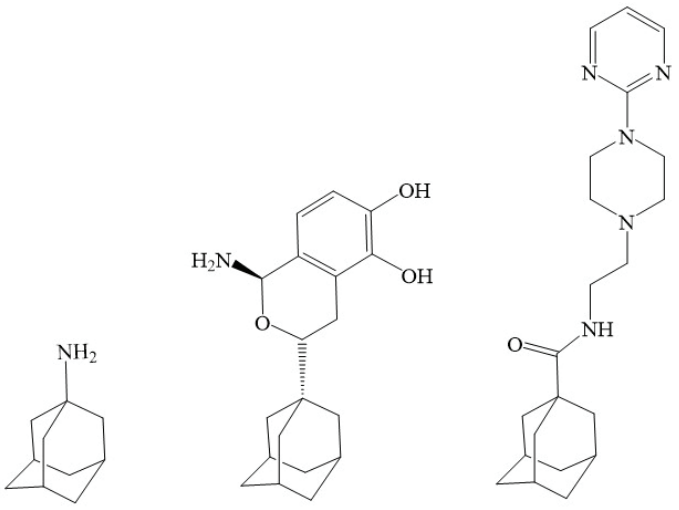
También se conoce que el núcleo adamantil está presente en varios agentes neuroactivos. De hecho, el compuesto **15** es un potente agonista de los receptores del tipo D_1 de la dopamina, de acción oral, por lo que es considerado un potencial agente para el tratamiento de la enfermedad de Parkinson. El adatanserin (**16**) es un antagonista mixto de los receptores de la serotonina $5HT_{1a}$/$5HT_{2a}$, y en la actualidad es un potencial candidato para los ensayos clínicos de Fase II como un agente ansiolítico [41] (Fig. 7). Por tales motivos, el diseño híbrido

del compuesto (**7**) como derivado quinolínico está orientado a un novel compuesto con actividad sobre los sistemas dopaminérgicos y/o serotoninérgicos centrales. Los estudios farmacológicos conductuales, nos muestran que tanto el compuesto **7** como el compuesto **10** actúan posiblemente a nivel presináptico aumentando la liberación de dopamina endógena y sobre el receptor $5HT_{1a}$ ejerciendo actividad agonística, ya que los resultados farmacológicos encontrados validan que el fragmento adamantil-metilamino es el responsable de esta actividad. Sin embargo, el compuesto **7**, mostró actividad agonística hacia el receptor $5HT_{2c}$, y esta selectividad se debe a la presencia del fragmento quinolil-metilamino. Por otro lado, la actividad dopaminérgica mostrada por ambos compuestos probablemente proviene de una acción indirecta (a nivel presináptico), y no por la activación de algún receptor dopaminérgico postsináptico, ya que ningún compuesto estudiado posee dentro de sus estructuras los fragmentos farmacóforicos de los receptores D_1 (fragmento 3,4-dihidroxi-feniletilamino) y D_2 (fragmento meta-hidroxi-feniletilamina).

Este estudio tiene algunas limitaciones. La primera es que carece de los estudios específicos para establecer los mecanismos moleculares de la acción farmacológica, de ensayos *in vitro* de unión a receptores y de estudios acerca de los mecanismos de señalización. Se requerirán estudios más amplios para establecer con mayor certeza la acción farmacológica de los compuestos sintetizados. Serán necesarios ensayos adicionales en el futuro para determinar los mecanismos que median la acción de estos nuevos compuestos. En segundo lugar, aun cuando el número de animales utilizado por grupo experimental luce como una posible limitación del estudio, cabe destacar que la tendencia actual es de usar el menor número posible de ratas en experimentos, de acuerdo con el principio de las tres erres**:** reemplazar, reducir y refinar.

La reducción implica estrategias para disminuir la cantidad de animales necesarios sin comprometer la validez de los resultados, mediante la aplicación de técnicas estadísticas avanzadas. Finalmente, los resultados son inferencias de los datos observados y se reconoce que los compuestos sintetizados y perfilados son apenas candidatos con potencial acción antipsicótica y antiparkinsoniana cuya confirmación requiere de estudios adicionales.

En conclusión, la evaluación farmacológica conductual demostró que los compuestos **7** y **10** a las dosis evaluadas actúan de manera indirecta sobre las vías dopaminérgicas presinápticas y al mismo tiempo sobre el sistema serotoninérgico. El compuesto (**7**), posee actividad agonística sobre los receptores $5HT_{2c}$, lo que abre la posibilidad de ser un novedoso candidato para el tratamiento de las enfermedades neuropsiquiátricas. Así mismo se demostró que el fragmento "adamantil-metilamino" contenido en los compuestos **7** y **10** proporciona la actividad serotoninérgica sobre el receptor $5HT_{1a}$, lo que sugiere que el compuesto **10** podría ser un candidato prometedor para tratar las enfermedades neurodegenerativas.



14 **15** **16**

Fig. 7. Compuestos **14 – 16**.

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Conflicto de interés

No existe ningún conflicto de interés.

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Contribución de los autores

MMR, JDG, MVV y AEA realizaron la parte experimental, tanto de síntesis como farmacológica. LBA, MMR, BCM y JEA diseño, síntesis y evaluación farmacológica del compuesto. ABC, JEC, MRG, AI y SEL participaron activamente en la toma de los espectros del compuesto, obtención de los animales para la parte de la evaluación farmacológica, ayuda económica, colaboración en la revisión del manuscrito y correcciones iniciales y la traducción en el caso que se ameritaba del manuscrito.

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Experimental study on the regulatory effect of Qinggan Dongyin on T lymphocyte homeostasis in MRL/lpr mice.

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Keywords: lupus erythematosus, systemic, Qinggan Dongyin, T-lymphocytes, signal pathways, cGAS-STING protein, mouse.

Abstract. Systemic lupus erythematosus (SLE) is an autoimmune disease marked by autoantibody overproduction and increased infection risk, even with current treatments. Dysregulated T lymphocyte homeostasis contributes to SLE progression, prompting exploration of immunomodulatory therapies. This study evaluated the effects of Qinggan Dongyin (QGDY), a compound of traditional Chinese medicine, in a murine SLE model. Twelve female MRL/lpr mice were randomly divided into model and QGDY treatment groups (n=6 each), with age-matched C57BL/6 mice as controls. QGDY (5 mL/kg/day) was administered via gavage for two weeks; controls received saline. Flow cytometry analyzed T cell subsets (CD4+, CD8+, Treg, Th1, Th2, Th17), ELISA measured plasma cytokines (IFN-γ, IL-6, TNF-α, IL-17A, TGF-β), HE staining assessed lung and kidney pathology, and qPCR evaluated cGAS and STING expression. Compared to the model group, QGDY significantly restored T cell balance by increasing CD4+, CD8+, and Treg cells and reducing Th1, Th2, and Th17 cells ($p<0.01$). QGDY also lowered pro-inflammatory cytokine levels ($p<0.05$), improved organ histopathology, and normalized elevated cGAS and STING expression ($p<0.01$). These findings indicate that QGDY exerts immunomodulatory effects in SLE, suggesting therapeutic potential through the regulation of T cell function and inflammatory signalling pathways.

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Estudio experimental sobre el efecto regulador de Qinggan Dongyin en la homeostasis de los linfocitos T en ratones MRL/lpr.

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Palabras clave: lupus eritematoso sistémico; Qinggan Dongyin linfocitos T; vías de señalización; proteínas cGAS y STING de ratón.

Resumen. El lupus eritematoso sistémico (LES) es una enfermedad autoinmune caracterizada por la sobreproducción de autoanticuerpos y un aumento del riesgo de infección, incluso con los tratamientos actuales. La homeostasis desrregulada de los linfocitos T contribuye a la progresión del LES, lo que impulsa la exploración de terapias inmunomoduladoras. Este estudio evaluó los efectos de Qinggan Dongyin (QGDY), un medicamento tradicional chino compuesto, en un modelo murino de LES. Doce ratones hembras MRL/lpr fueron divididas aleatoriamente un grupo modelo y un grupo de tratamiento con QGDY (n=6 en cada grupo), con ratones C57BL/6 emparejados por edad como controles. Se administró QGDY (5 mL/kg/día) por medio de sonda durante dos semanas; los controles recibieron solución salina. Se analizó la subpoblación de células T (CD4+, CD8+, Treg, Th1, Th2, Th17) mediante citometría de flujo, se midieron las citoquinas plasmáticas (IFN-γ, IL-6, TNF-α, IL-17A, TGF-β) por ELISA, se evaluó la patología pulmonar y renal mediante tinción con HE, y se evaluó la expresión de cGAS y STING mediante qPCR. En comparación con el grupo modelo, QGDY restauró significativamente el equilibrio de las células T al aumentar las células CD4+, CD8+ y Treg y reducir las células Th1, Th2 y Th17 ($p<0,01$). QGDY también disminuyó los niveles de citoquinas proinflamatorias ($p<0,05$), mejoró la histopatología de órganos y normalizó la expresión elevada de cGAS y STING ($p<0,01$). Estos hallazgos indican que QGDY ejerce efectos inmunomoduladores en el LES, sugiriendo un potencial terapéutico a través de la regulación de la función de las células T y las vías de señalización inflamatoria.

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder marked by the overproduction of autoantibodies and the formation of immune complexes, which can impact various organs and systems [1]. While advancements in diagnosis and treatment have enhanced the survival rate of SLE patients, the mortality rate remains high [2]. Infections, particularly in the context of the recent global spread of SARS-CoV-2, have emerged as a significant cause of death among SLE patients [3]. Research indicates that SLE patients face significantly higher risks compared to the general population [4-5].

In autoimmune diseases such as SLE, infection and autoimmune response interact with each other. On the one hand, infection can destroy the immune tolerance to autoantigens and induce the development of autoimmune diseases in individuals with a genetic predisposition. The persistent infection of pathogens may lead to the aggrava-

tion of disease activity via molecular simulation, bystander activation, epitope diffusion or polyclonal activation [6]. On the other hand, high disease activity, immune dysregulation, and drugs (such as glucocorticoids and immunosuppressants) induce immune deficiency and organ failure [2].

Lymphocytes are key players in the pathogenesis of SLE [7,8]. Dysregulation of T lymphocytes, including changes in cell count, subset distribution, and function, is implicated in the progression of SLE and is closely linked to infection risk [9]. Specifically, a reduction in CD4+ T cells is the most frequently reported hematological abnormality in SLE, often correlating with a higher risk of infection [10]. Meanwhile, CD8+ T cells, which are cytotoxic and typically destroy target cells through the release of perforin and granzyme, show impaired function in SLE patients. This dysfunction leads to increased risks of both infections and autoimmune reactions [11]. In addition, Treg, Th1, Th2, and Th17 cells are important subsets of CD4+ T lymphocytes, and their distribution and function are significantly disordered. The absolute decrease in the number of Tregs is associated with immune tolerance disruption and the subsequent worsened disease activity and increased infection risk in SLE patients [12]. Meanwhile, the increase in Th cell activity may enhance the local tissue inflammation and damage important target organs such as the kidneys [13-15]. Therefore, regulation of the T lymphocyte homeostasis in SLE patients may effectively control the autoimmune response while maintaining immune function and preventing infection, hopefully providing an effective treatment method to meet the urgent clinical need.

Qinggan Dongyin (QGDY) is a compound preparation consisting of multiple Chinese herbal medicines, it was developed based on the experience of Academician Zhang Boli's team in the prevention and control of a novel coronavirus infection in Wuhan [16], and the main therapeutic goal of this prescription is to protect vital qi (defined as enhancing the body's defenses and promoting recovery in traditional Chinese medicine), resist exopathogens, and clear away heat and toxic material. Clinical studies have shown that QGDY is highly effective in preventing respiratory tract infections with a favorable safety profile [16]. Network pharmacological studies have shown that QGDY plays a regulatory role in various viral infections by regulating the immune-inflammatory response caused by infection[17]. Pharmacological studies have also found that QGDY has antioxidant and anti-inflammatory activities, and significantly regulates the number of lymphocytes, the level of interleukin and the release of TNF-α in in peripheral blood [18-19]. The usefulness of QGDY in infections has been reported, but the experience with this substance in autoimmune diseases has not. In the present study, the SLE mouse model (MRL/lpr mouse) was intervened with QGDY. The distribution of T lymphocyte subsets, expression of related cytokines, and pathological damage of lung and kidney in these mice were observed to clarify the regulatory effects of QGDY on T lymphocyte homeostasis and immune response in MRL/lpr mice, providing supportive data for adjuvant treatment of SLE and risk reduction of infection.

MATERIALS AND METHODS

Instruments and reagents

Main instruments included BD FACSCalibur flow cytometer (BD, USA), ELX800 microplate reader (BioTek, USA), inverted microscope (Nikon TS2), desktop high-speed refrigerated microcentrifuge (D3024R, DLAB Scientific Co., Ltd), and fluorescence quantitative PCR instrument (Stepone plus, ABI, USA).

Main reagents included FITC anti-mouse CD3 antibody (item number: E-AB-F1013C), PE anti-mouse CD4 antibody (item number: E-AB-F1097D), PerCP anti-mouse CD8a antibody item number: E-AB-F1104F), APC anti-mouse Foxp3 antibody (item

number: E-AB-F1238E), APC anti-mouse CD183/CXCR3 antibody (item number: E-AB-F1114E), APC anti-human/mouse CD44 antibody (item number: E-AB-F1100E), APC anti-mouse IL-17A antibody (item number: E-AB-F1272E), all antibodies were purchased from Elabscience. Other reagents were hematoxylin (Servicebio, item number: G1004-100ML), eosin staining solution (Solarbio, item number: G1108), mouse interferon gamma (IFN-γ) ELISA kit (Meimian, item number: MM-0182M2), mouse IL-6 ELISA kit (Meimian, item number: MM-0163M2), mouse TNF-α ELISA kit (Meimian, item number: MM-0180R2), mouse IL-17A ELISA kit (Meimian, item number: MM-0180R2), mouse TGF-β ELISA kit (Meimian, item number: MM-0689M2), mouse PFP ELISA kit (Meimian, item number: MM-4504M2), Trizol reagent (Vazyme, item number: R401-1), and MonAmp™ SYBR® Green qPCR Mix (Wuhan Monad Biotechnology Co., Ltd., item number: MQ10201S).

Experimental animals and grouping

Twelve female MRL/lpr mice, aged eight weeks, were randomly assigned to two groups: the model group and the QGDY group, with six mice in each. Additionally, six age-matched female C57BL/6 mice were used as the control group. All mice were purchased from SPF (Beijing) Biotechnology Co. Ltd. The mice were housed in a room with natural lighting, maintained at a temperature of 20-24°C, with proper ventilation and humidity control. After a 1-week acclimatization period with free access to food and water, the experiment commenced once the mice were in optimal health. The study was approved by the Animal Ethics Committee (Approval No. IRM/IRM/2-IACUC-2403-126).

Preparation and concentration of QGDY

QGDY consisted of *Astragalus membranaceus* 18g, *Polygonum cuspidatum* 18g, stir-baked *Fructus arctii* 18g, *Belamcanda sinensis* 12g, *Platycodon grandiflorum* 12g, *Radix paeoniae rubra* 12g, *Perilla frutescens* leaves 12g, honeysuckle 18g, charred hawthorn 12g, and licorice 6g. The dosage was calculated based on the "Table of equivalent dose ratios between humans and animals by body surface area" [1]. 0.3588g, i.e., 17.94g/kg, of QGDY crude drug, was required for a 20g mouse per day, assuming that the coefficient between humans (70kg) and mice (20g) was 0.0026 and that 138g of the crude drug was needed for each patient daily. All medicinal herbs were boiled twice, and the decoction was combined, filtered, and concentrated to obtain a medicinal solution containing 3.6g/mL of crude drug. Animals in the QGDY group were given QGDY by gavage at a dose of 5mL/kg, while those in the control group and the model group were given normal saline by gavage at a dose of 5ml/kg for two consecutive weeks, once a day. Gavage was administered at a fixed time (9:00-10:00 am) every day without fasting.

Sample collection and processing

After two weeks of intragastric administration, mice in each group were anesthetized with 1% pentobarbital sodium to reduce their stress response. Plasma was collected, and the mice were then sacrificed to collect corresponding samples. (1) Plasma was collected. Briefly, the blood was collected from the eyeballs of mice, and placed in a centrifuge tube containing EDTA for anticoagulation at RT for two hours, followed by centrifugation at 3500rpm for 15 minutes. The supernatant was collected and frozen at −80℃ in an EP tube for ELISA. (2) Following euthanasia, the abdominal cavity was opened to remove the lungs. The left lung was fixed in 4% paraformaldehyde for histopathological analysis, while the right lung was collected for PCR testing. (3) After sacrificing the mice with various treatments, the abdominal cavity was opened to extract the left kidney, which was fixed in 4% paraformaldehyde for pathological examination. In contrast, the right kidney was stored at −80℃ for potential future analysis or as a

backup sample. Additionally, the spleen was removed and placed in PBS for subsequent flow cytometry analysis.

Testing indexes and methods

Flow cytometry

Splenic tissue was sliced, ground and filtered, and the tissue filtrate was used to isolate monocytes. Briefly, an appropriate amount of separation solution of Lymphoprep (AN1001967) from Shanghai Shanjin Biotechnology Co., Ltd (Shanghai, China) was added to the centrifuge tube, and the diluted tissue filtrate was placed on top of the separation solution, with attention to keep the interface clear between the two liquid layers. The tube was centrifuged using a horizontal rotor at 500 x g for 20 minutes at RT. After centrifugation, layers were seen: the top layer contained the diluted plasma, the middle layer was the transparent separation solution, with a white membrane layer between the plasma and the separation solution, which was the lymphocyte layer, and the red blood cells and granulocytes gathered at the bottom of the centrifugation tube. Cells in the white membrane were carefully removed and placed in a clean 15mL centrifuge tube, and washed with 10 mL PBS, followed by centrifugation at 250×g for 10 minutes. The supernatant was discarded, 5mL PBS was added to resuspend the cells, and the cells were centrifuged at 250 × g for 10 minutes. This step was repeated twice. After centrifugation, the supernatant was discarded, and the cells were resuspended. 1×10^5 cells and 100μL (1 ×) Binding Buffer were added to each sample tube, and staining was performed. Cells were first washed twice with PBS. Subsequently, Cyto-Fast Fix/Perm Buffer was added to fix the cells, which were then incubated at 4℃ for 20 minutes before being rewashed with PBS. APC-conjugated anti-mouse Foxp3 antibody (ab215206) for Treg cells, CD4 antibody (ab237722) for CD4+ T cells, CD8 antibody (ab237709) for CD8+ T, IFN-γ antibody (ab280353) for Th1 cells, IL-4 antibody (ab225638) for Th2 cells, and IL-17A antibody (ab302922) for Th17 cells from Abcam biotechnology company (UK) was added and incubated for 15 minutes. An additional 400 μL of 1x Binding Buffer was added to each tube, followed by filtration of the mixture. A total of 10,000 cells were collected from the stained samples using a BD FACSCalibur flow cytometer.

ELISA

Plasma levels of IFN-γ (H025-1-2), IL-17A (H014-2), TGF-β (H034-1-1) were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China), and the IL-6 (ab222503, Abcam), TNF-α (ab183218, Abcam), and perforin protein (PFP) (ab114201, Abcam) were obtained from Abcam biotechnology company for inflammatory factor detection using ELISA kits following the manufacturer's protocols. The OD of each well was detected with an ELX800 microplate reader at a wavelength of 450 nm.

HE staining

Lung and kidney tissues fixed in 4% paraformaldehyde underwent dehydration, clarification, paraffin embedding, and sectioning. Following HE staining, the pathological changes in the lungs and kidneys were examined for each group (control group, model group, and QGDY group), under a light microscope at magnifications of 200x (top row) and 400x (bottom row). For the histological parameters, the inflammatory cell infiltration (neutrophils and lymphocytes), alveolar structure changes (Alveolar wall thickness, alveolar cavity, and interstitial fibrosis) were examined in lung tissues, while the vacuoles, glomerular consolidation, glomerular structure change, inflammatory cell infiltration, and cell proliferation were examined in kidney tissues.

Quantitative PCR (qPCR)

Total RNAs were extracted. Briefly, tissues, adherent cells, and whole blood sam-

ples were pretreated and lysed using an RNA extraction solution, followed by centrifugation and precipitation of RNA. The concentration and purity of RNAs were determined. Next, reverse transcription was performed. A reverse transcription reaction system containing a gDNA digestion mixture and Hifair® III SuperMix was prepared, and the reaction program was set (25℃, 55℃, and 85℃). The quantitative PCR was conducted. A PCR reaction system was prepared using MonAmp™ SYBR® Green qPCR Mix and primers, and temperature cycles, including pre-denaturation, denaturation, annealing, elongation, and melting curves, were set. Finally, the fold expressions of the target genes were calculated using the 2^-ΔΔCt method to evaluate their relative expression levels.

The sequences of primers used included: Primers for inflammation-related genes of cyclic GMP-AMP synthase (cGAS): forward (cGAS-F): TATGGCGGTGACACACTTCC, and reverse (cGAS-R): GTCAGGACAGGTGAGCAGAC; primers for mouse stimulator of interferon gene (STING): forward (STING-F): TGTCTGGCTGAAGAGCTGTG, and reverse (STING-R): CGATTCTTGATGCCAGCACG.

Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistics 26 software. Quantitative data were expressed as mean±standard deviation, while measurement data were reported as median (interquartile range) [M (IQR)]. For comparisons between groups, normally distributed data were analyzed using one-way ANOVA followed by Tukey's test, whereas non-normally distributed data were evaluated using the non-parametric Kruskal-Wallis test, $p<0.05$ was considered statistically significant.

RESULTS

Distribution of immune cells and Treg lymphocytes in the spleens of mice in each group

Significant differences were observed in the expression levels of various lymphocyte subsets between the groups. The model group exhibited a notable decrease in CD4+ T, CD8+ T, and Treg cells, while Th1, Th2, and Th17 lymphocytes were elevated ($p<0.01$). In contrast, the QGDY group showed a reversal of these trends, with increased levels of CD4+ T, CD8+ T, and Treg cells and decreased levels of Th1, Th2, and Th17 lymphocytes ($p<0.01$) (Table 1).

Concentrations of plasma IFN-γ, IL-6, TNF-α, IL-17A, TGF-β and PFP in each group of mice

Significant changes in plasma cytokine levels were observed between the groups. The

Table 1. Distribution of CD4 + T, CD8 + T, Th1, Th2, Th17 and Treg cells in the spleen of mice in each group.

	Control group (n=6)	Model group (n=6)	QGDY group (n=6)
CD4+ T (%)	19.60 ± 1.05	15.40 ± 0.30***	17.60 ± 0.57###
CD8+ T (%)	12.65 ± 1.10	6.33 ± 0.46***	9.52 ± 0.19###
Th1 (%)	0.85 ± 0.26	3.40 ± 0.51**	1.60 ± 0.11##
Th2 (%)	0.76 ± 0.12	3.26 ± 0.71***	1.58 ± 0.05###
Th17 (%)	1.34 ± 0.34	2.86 ± 0.16***	1.95 ± 0.15###
Treg (%)	2.59 ± 0.14	0.59 ± 0.26**	1.61 ± 0.08##

Note: Cells of CD4-positive T cells (CD4+ T), CD8-positive T cells (CD8+ T), T-helper 1 cells (Th1), T-helper 2 cells (Th2), T-helper 17 cells (Th17), Regulatory T cells (Treg) in model and treatment group compared with the control group using one-way ANOVA method, and the mean ± standard deviation (SD) is used for the result representation. **p<0.01, ***p<0.001; compared with the model group, ###p<0.001, ##p<0.01.

model group exhibited higher levels of IFN-γ, IL-6, TNF-α, IL-17A, and TGF-β ($p<0.05$), while the level of PFP was lower ($p<0.05$). In the QGDY group, the levels of IFN-γ, TNF-α, IL-17A, and TGF-β were reduced compared to the model group ($p<0.05$) (Table 2).

Pathological analysis of lung and kidney tissues in each group of mice

In the control group, the pulmonary alveoli were evenly distributed with neatly arranged cells, the alveolar wall was thin and uniform, and the alveolar cavity was clear and visible. In contrast, the model group exhibited disordered cell arrangement, thickened alveolar walls and narrowing or even occlusion of the alveolar cavities, increased lung parenchyma, and more severe inflammation. The QGDY group showed improved cell arrangement, reduced lung parenchyma, and less inflammation compared to the model group (Fig. 1).

Table 2. Concentrations of IFN-γ, IL-6, TNF-α, IL-17A, TGF-β and PFP in the plasma of mice in each group.

	Control group (n=6)	Model group (n=6)	QGDY group (n=6)
IFN-γ (ng/L)	120.56 ± 42.44	461.76 ± 304.88*	168.99 ± 36.32#
IL-6 (pg/mL)	31.05 ± 11.39	86.16 ± 71.44*	28.28 ± 45.16
TNF-α (ng/L)	80.94 ± 46.70	385.03 ± 238.84**	189.11 ± 204.82#
IL-17A (pg/mL)	20.26 ± 6.09	105.87 ± 46.26**	45.99 ± 42.64#
TGF-β (ng/L)	24.53 ± 24.68	190.48 ± 148.21**	62.24 ± 40.51#
PFP (ng/L)	49.83 ± 24.07	11.34 ± 7.53**	31.21 ± 17.88

Note: The cytokines of Interferon-gamma (IFN-γ), Interleukin-6 (IL-6), Tumor Necrosis Factor-α (TNF-α), Interleukin-17A (IL-17A), Transforming Growth Factor-beta (TGF-β), Perforin (PFP) in model and treatment groups compared with the control group using One-way ANOVA method, and the mean ± standard deviation (SD) is used for the result representation. *$p<0.05$, **$p<0.01$; compared with the model group, #$p<0.05$.

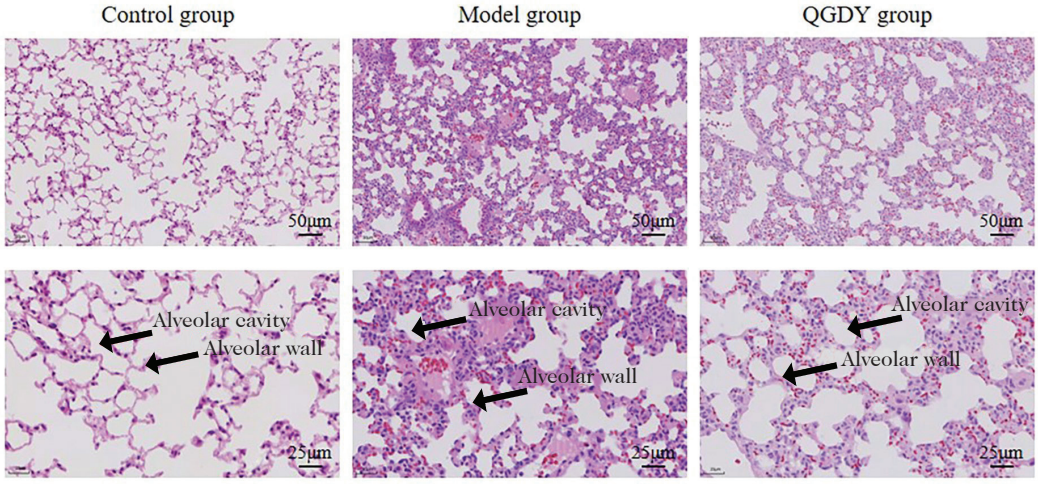

Fig. 1. Histopathological changes in lung tissues of different groups (HE staining).

Inflammation was defined as the presence of increased numbers of inflammatory cells (primarily neutrophils and mononuclear cells) within the alveolar spaces and interstitial areas.

Overall, the degree of inflammation was assessed by evaluating the density and distribution of these cells. The control group showed minimal to no inflammation, while the model group exhibited significant infiltration of inflammatory cells. The QGDY group showed a reduction in inflammatory cell infiltration compared to the model group.

Regarding kidney pathology, the control group had intact glomerular morphology with minimal inflammatory cell infiltration. The model group displayed glomerular consolidation, vacuoles, proliferation, and significant inflammatory cell infiltration. The QGDY group had more orderly glomerular structures and reduced inflammation compared to the model group (Fig. 2).

Expression of cGAS and STING

Expression levels of cGAS and STING varied significantly among the control, model, and QGDY groups ($p<0.01$). In the model group, elevated cGAS and STING expressions were noted compared to the control group, reflecting heightened immune activation. Conversely, the QGDY group exhibited reduced cGAS and STING levels, nearing those of the control group, implying that QGDY might modulate this critical immune signalling pathway (Fig. 3).

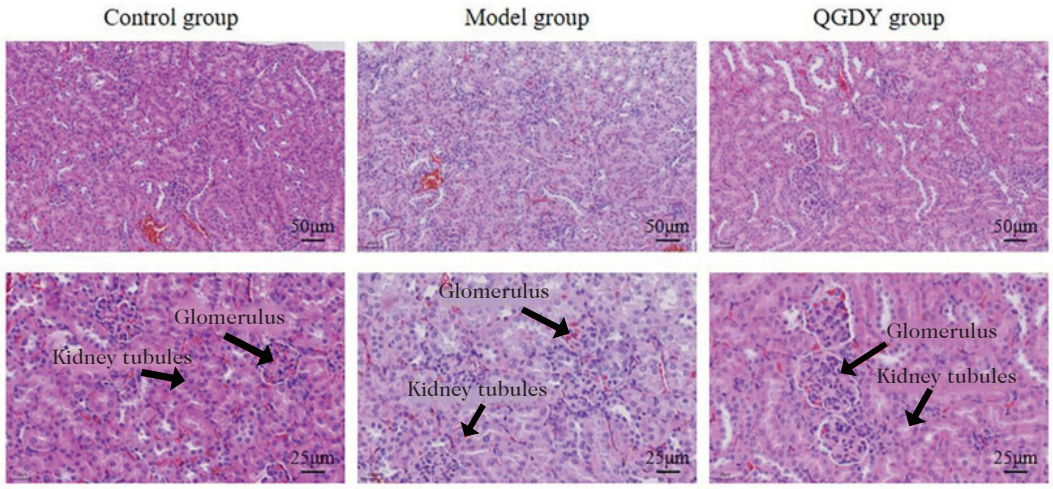

Fig. 2. Pathological analysis of kidney tissue in each group of mice.

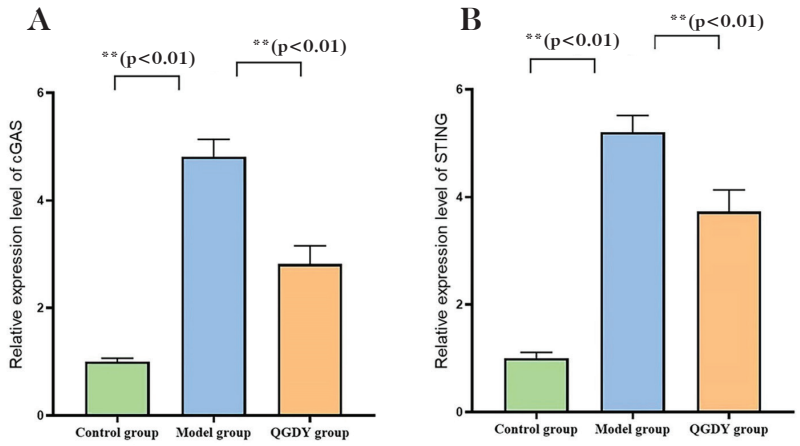

Fig. 3. qPCR for the expression of cyclic GMP-AMP synthase (cGAS) and stimulator of interferon gene (STING). (A) The relative expression level of cGAS, and (B) the relative expression level of STING.

DISCUSSION

The regulatory effect of QGDY on T lymphocyte homeostasis and related immune signalling pathways in MRL/lpr mouse models was investigated in the present study. These model animals have characteristics similar to human SLE due to spontaneous mutations in the *lpr* gene (lymphoproliferation gene), including lymphocyte abnormalities, lymph node enlargement, excessive autoantibodies and glomerulonephritis [20], making it a classic model for studies on the immunopathological mechanism of SLE. The abnormal distribution and dysfunction of T lymphocyte subsets are one of the key factors in the pathogenesis of SLE[21]. Previous studies have revealed significant changes in the number of T subsets in MRL/lpr mice [22]. It was found in the present study that QGDY significantly improved the distribution of T lymphocytes in MRL/lpr mouse models, enhanced the expression of CD4+, CD8+ and Treg cells, and reduced the number of Th1, Th2 and Th17 cells. This prescription also effectively inhibited the levels of plasma inflammatory factors IFN-γ, IL-6, TNF-α, IL-17A and TGF-β, indicating its anti-inflammatory effect. The pathological evaluation showed that QGDY reduced the inflammation and damage to the lung and kidney and maintained the integrity of tissue structure. In addition, QGDY alleviated immune activation by regulating the cGAS and STING signalling pathways.

The findings offer fresh evidence supporting the potential use of QGDY in treating SLE. This study investigated the impact of QGDY on T cell function in MRL/lpr mice to elucidate its possible protective role in SLE. Dysfunctional T cells can elevate infection risk, particularly during immunosuppressive treatment. In SLE patients, compromised CD4+ T cell function not only affects autoantibody production but also weakens infection defense. Moreover, impaired CD8+ T cell function further exacerbates the infection risk [23]. Regulating T cell function is crucial for enhancing the autoimmune response and preventing infections in SLE. Our study demonstrated that QGDY significantly boosted the expression of CD4+ and CD8+ T cells, thereby strengthening the immune response to infections and lowering infection risk.

Additionally, QGDY reduced the number of Th1, Th2, and Th17 cells while increasing Treg cell expression. These changes are significant because Th1 and Th17 cells are often overactivated in SLE patients, driving inflammation through IFN-γ and IL-17 secretion and leading to tissue damage [24]. Thus, QGD's anti-inflammatory effects may mitigate SLE pathology by modulating the activity of these cells. Moreover, the cGAS-STING signalling pathway's role is noteworthy. QGDY significantly downregulated cGAS and STING expression, suggesting it curbs inflammation by inhibiting this pathway's overactivation. This mechanism likely impacts T lymphocyte differentiation and function, improving SLE's pathological state. Since the cGAS-STING pathway is linked to IFN-I production [25], the observed a reduction in related cytokines after QGDY intervention further supports its immune-regulating potential.

The high expression levels of TNF-α and IFN-γ are associated with the activation of Th1 lymphocytes and tissue damage [26]. The levels of plasma IFN-γ and TNF-α significantly decreased after treatment with QGDY, indicating the ability of this prescription to inhibit the activation of Th1 cells and alleviate the inflammation in involved organs. The increase of IL-6 and IL-17 is associated with Th17-mediated inflammatory response [27]. The changes in TGF-β expression may affect the function and quantity of Tregs and consequently, the immune tolerance. It was found in the present study that QGDY also significantly reduced the levels of these two cytokines, suggesting that it may reduce the inflammatory response via regulating the activity of Th17 cells. The changes in TGF-β level may affect the function and quantity of

Tregs [28], and the results of the present study showed that QGDY effectively enhanced the expression of Tregs, providing a basis for the improvement of immune tolerance. Pathological evaluation of the lungs and kidneys of mice demonstrated that QGDY significantly reduced tissue damage in MRL/lpr mice. The disordered cell arrangement and significant inflammation observed in the models were recovered to an improved cell structure and reduced inflammation after treatment with QGDY, indicating that QGDY can improve the pathological changes related to SLE by regulating T cell function and reducing inflammatory factors, providing new ideas for SLE treatment.

Modern studies have found that the components of QGDY are rich in various bioactive substances, including flavonoids, organic acids, and monoterpenes, which exert multiple regulatory effects on the human immune system. The medicinal herbs in QGDY, such as *Astragalus membranaceus* and honeysuckle, can enhance immunity and prevent inflammation [29], which are in line with the treatment goals of SLE. Progress has been made on the effects of individual medicinal herbs in QGDY on the function of T lymphocytes. For example, *Astragalus membranaceus* has been found to enhance the proliferation and differentiation of T lymphocytes and improve the immunity of body [29]. The active compounds contained in medicinal herbs such as *Polygonum cuspidatum* and stir-baked *Fructus arctii* regulate the cytokines produced by T lymphocytes and affect the function of these cells. Medicinal herbs such as *Belamcanda sinensis* and *Platycodon grandiflorum* have shown the potential to regulate the balance of T subsets, which can help improve the overactive state of the immune system. *Radix paeoniae rubra*, *Perilla frutescens* leaves, honeysuckle, charred hawthorn and Licorice are also involved in the regulation of T cell-mediated immune responses.

There are limitations in the present study. First, although the MRL/lpr mouse is a classical animal model, it cannot completely simulate the complex pathological process of human SLE, which may affect the clinical translation of the results. Second, the sample size was relatively small. In addition, the specific effects of individual components of QGDY on T cell subsets and the underlying mechanism(s) were not adequately explored, and an in-depth molecular investigation was not conducted. Also, the study focused mainly on short-term treatment effects and long-term safety and efficacy were not evaluated. Finally, clinically relevant indicators such as improvement in clinical symptoms, quality of life of patients, and biomarkers were not evaluated. These limitations reminded us that caution should be exercised in interpreting the findings and providing directions for improvement in subsequent studies. Future studies with samples from broad and diverse sources are needed for long-term efficacy evaluation and in-depth discussion of mechanisms further to verify the potential application of QGDY in clinical practice.

QGDY significantly improves T lymphocyte homeostasis, reduces the levels of inflammatory factors, and alleviates organ pathological damage in MRL/lpr mice. Meanwhile, this prescription effectively regulates the expression of immune-related signalling molecules such as cGAS and STING, making it a promising adjuvant therapy for SLE to reduce the risk of infection via regulating immune balance, and providing new ideas and data support for clinical treatment of SLE.

Ethics approval and consent to participate

This study was conducted following the principles of ethical animal research outlined in the Basel Declaration and the ethical guidelines by the International Council for Laboratory Animal Science (ICLAS). This study was conducted under the NC3Rs ARRIVE guidelines.

The experimental protocol was approved by the Institute of Radiation Medicine, Chinese Academy of Medical Sciences

and the Peking Union Medical College (Approval Number: IRM/2-IACUC-2403-126). Experimental animals underwent all procedures under anesthesia, and every effort was made to minimize their pain, suffering, and death.

Data availability statement

All data generated or analyzed during this study are included in this article.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Participation of each author

NJ and XC conceived of the study, HH, HX and SW participated in its design and coordination, JL and SZ helped to draft the manuscript. All authors read and approved the final manuscript.

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MiR-451 ameliorates red blood cell storage damage and macrophage polarization-mediated transfusion immunity by regulating the AMPK/mTOR signalling pathway.

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Keywords: microRNA miR-451; ATF2; AMP-activated protein kinases/mTOR pathway; erythrocytes; blood preservation macrophages.

Abstract. Red blood cells (RBCs) undergo a series of structural and functional changes during storage, and miR-451 is crucial for maintaining homeostasis of RBCs. Inflammatory factors and miR-451 expression in whole blood at different storage times were detected by ELISA and qRT-PCR. THP-1 cells were induced into M0 macrophages. Subsequently, miR-451 mimics, miR-451 inhibitor, and activating transcription factor 2 (ATF2) were transfected into the cells, followed by the application of compound C. Macrophages were then polarized to the M1 phenotype. Macrophage markers were discovered through flow cytometry and Western blot. The adenosine monophosphate-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) pathway protein levels were detected using Western blot. Finally, a mouse model of traumatic hemorrhagic shock was constructed, and blood transfusion and tail vein injection of agomir-451 were performed. The levels of M1 macrophage markers and inflammatory factors were detected by flow cytometry and ELISA, respectively. When the human whole blood storage time was 21 d and 35 d, the expression of miR-451 decreased, and the proinflammatory factor contents increased. When miR-451 was overexpressed, proinflammatory cytokines and M1 macrophage markers' expression in THP-1 cells were reduced, p-AMPK level was increased, and p-mTOR level was decreased. After overexpression of ATF2 or compound C, proinflammatory factors and M1 macrophage markers in THP-1 cells increased, and p-AMPK and p-mTOR expression reversed. Overexpression of miR-451 also inhibited macrophage M1 polarization and inflammation in shock mice. miR-451 inhibits ATF2-regulated AMPK/mTOR pathway, inhibits macrophage M1 polarization and inflammation, and improves RBC storage damage.

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MiR-451 mejora el daño de almacenamiento de glóbulos rojos y la inmunidad de transfusión mediada por la polarización de macrófagos mediante la regulación de la señalización AMPK/mTOR.

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Palabras clave: micro ARN miR-451; ATF2; proteínas quinasa activadas por AMP/mTOR; eritrocitos; conservación de la sangre; macrófagos.

Resumen. Los glóbulos rojos (RBC) sufren una serie de cambios estructurales y funcionales durante el almacenamiento, y miR-451a es crucial para la homeostasis de los glóbulos rojos. MiR-451 puede mantener la homeostasis de los glóbulos rojos. Los factores inflamatorios y la expresión de miR-451 en sangre completa a diferentes tiempos de almacenamiento fueron detectados por ELISA y qRT-PCR. Las células THP-1 fueron inducida a macrófagos M0, luego el miR-451 imitador y el miR-451inhibidor, junto al factor de activación de transcrpción 2 (ATF2) se transfectaron a las células, seguido de la aplicación del compuesto C. Los macrófagos son entonces polarizados a tipo M1. Los marcadores de macrófagos fueron evaluados mediante citometría de flujo y Western blot. Los niveles de proteínas de la vía de la proteína quinasa-activada por monofosfato de adenosina (AMPK)/blanco de la rapamicina en mamíferos (mTOR) fueron detectados usando Western blot. Finalmente, se construyó un modelo de shock hemorrágico traumático en ratón , y se realizó transfusión sanguínea e inyección en vena de la cola de agomir-451. Los marcadores de macrófagos tipo M1 y los niveles de factores inflamatorios fueron examinados por citometría de flujo y ELISA, respectivamente. Cuando el tiempo de almacenamiento de sangre total humana fue de 21 y 35 días, la expresión de miR-451 disminuyó y el contenido de factor pro-inflamatorio aumentó. Cuando mir-451 se superexpresa, la expresión de citocinas proinflamatorias y marcadores de macrófagos de tipo M1 en las células THP-1 disminuye, los niveles de p-AMPK aumentan y los niveles de p-mTOR disminuyen. La superexpresión de ATF2 o la aplicación del compuesto C aumenta el factor proinflamatorio y los marcadores de macrófagos de tipo M1 en las células THP-1, y la expresión de p-AMPK y p-mTOR se revierte. La sobreexpresión de miR-451 también inhibe la la polarización M1 y la inflamación en macrófagos de ratones en shock. El miR-451 inhibe el ATF2 para regular la vía AMPK/mTOR, inhibe la polarización e inflamación por los macrófagos M1 y mejora el daño de almacenamiento de RBC.

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INTRODUCTION

Red blood cell (RBC) transfusion is a standard treatment that saves lives. The unique cell structure of RBCs enables them to have good deformability and the ability to transport O_2. This structure also makes RBCs highly susceptible to environmental influences and has a short lifespan, making them prone to harmful lesions *in vitro*. At

present, the primary storage method of RBC is to suspend them in different components of RBC preservation solution [1]. However, RBC storage is accompanied by a series of biochemical and morphological changes, which eventually lead to the decline of RBC quality and function, which is called RBC storage damage [2]. There may be a potential negative relationship between RBC storage time and blood transfusion outcome, which will reduce the effect of clinical blood transfusion treatment and may even lead to the occurrence of serious adverse events of blood transfusion [3]. Therefore, RBC storage damage has become a key area of blood transfusion safety. Prolonging RBC storage time and effectiveness, and obtaining higher quality RBCs to meet clinical blood use needs, is of great significance.

Studies have found that infusion of red blood cells with extended storage time can cause persistent lung inflammation and injury [5]. The extracellular vesicles produced by stored red blood cells can induce the production of tumor necrosis factor-α (TNF-α), interleukin (IL) -6 and IL-8, resulting in a strong inflammatory response in the recipient. The vesicles also contain a large amount of coagulation substances, which alter the recipient's coagulation function, potentially leading to adverse outcomes from red blood cell transfusion. In addition, the storage of red blood cells will produce a large amount of free iron ions, which will activate macrophages to release a large number of inflammatory substances after infusion into the human body, potentially leading to a systemic inflammatory response in the recipient [7]. It can be inferred that inhibition of inflammation is significant in improving red blood cell storage damage.

MicroRNA (miRNA) plays a role in regulating gene expression throughout biological evolution [8]. In 2010, for the first time, researchers reported the miRNA differential map of stored RBC, which provided a new idea for a comprehensive understanding of RBC storage damage, and proposed that there may be miRNA regulatory pathways in stored RBC [9]M. Later, researchers disclosed the protective impact of miR-196a on stored RBC decay [10], and the up-regulation of miR-203 expression can prolong the storage time of RBC by inhibiting apoptosis [11]. However, so far, little is known about the exact mechanism of abnormal expression of miRNAs in RBC storage damage. miR-451 is the only miRNA found so far that does not depend on the Dicer enzyme for maturation. miR-451 is the most expressed miRNA in mature RBC, and its abnormal expression is also associated with a variety of blood diseases [12]. In stored RBC, miR-451 is also abnormally up-regulated [13]. In conclusion, miR-451 may have an improvement effect on RBC storage damage.

miR-451 can regulate cell function through adenosine monophosphate-activated protein kinase (AMPK) / mammalian target of rapamycin (mTOR) pathway [14]. It is known that adenosine triphosphate (ATP) consumption and Ca^{2+} accumulation occur in stored RBCs. AMPK can activate and regulate downstream target phosphorylation through the above process [15]. The survival rate of RBC transfusion is negatively related to deformability-related phosphorylation [16]. mTOR is a highly conserved serine/threonine protein kinase. mTOR, as a key signalling pathway for cell metabolism regulation, can regulate biological macromolecules as cells' cornerstone [17]. In addition to the plasma membrane and cytoplasm, mature RBCs have no other organelles, cannot carry out aerobic oxidation of sugar, and can only use glycolysis for energy supply [18]. In addition to the plasma membrane and cytoplasm, mature RBCs have no other organelles, cannot carry out aerobic oxidation of sugar, and can only use glycolysis for energy supply. Inhibition of the mTOR signalling pathway can downregulate G-6-PD to regulate changes in cell biological function [20]. In recent years, studies have found that exposure to drugs such as mTOR inhibitors can affect RBC number and redox metabolism, affect the

quality of RBC storage and the efficacy after blood transfusion [21], suggesting that miR-451 may affect RBC metabolism by regulating AMPK/mTOR signalling and participate in RBC storage damage.

In summary, we propose a hypothesis that miR-451 regulates the AMPK/mTOR pathway and is vital for RBC storage damage and transfusion-related complications. In this study, the miR-451 level in long-term stored blood was detected, and then its expression was interfered with. The macrophage polarization and inflammatory response indexes were measured, and the role of the AMPK/mTOR pathway was explored. The goal of this study is to prove that miR-451 can regulate the AMPK/mTOR signalling pathway to improve red blood cell storage damage and provide a theoretical basis for improving RBC storage damage and blood transfusion complications.

METHODS

Sample source

Whole blood was obtained from 20 healthy donors. All donors have read and signed the informed consent. The Medical Ethics Committee of Jiaxing First Hospital has approved this study. Four hundred mL of whole blood was collected from each donor, and processed and stored according to the operation specifications of the blood station. Whole blood was collected in citrate-phosphate-glucose solution (CPD; 3 mg/mL citric acid, 26.30 mg/mL sodium citrate, 2.22 mg/mL sodium dihydrogen phosphate, and 25.51 mg/mL glucose), and a portion of the blood was taken out for immediate detection. The expression levels of inflammatory factors and miR-451 in the whole blood were detected by enzyme-linked immunosorbent assay (ELISA) and quantitative real-time polymerase chain reaction (qRT-PCR). The remaining blood was sub-packed in a 1.7 mL blood bag catheter and stored at 4 ℃ for up to 35 days.

Human monocytic leukemia THP-1 cells were purchased from Punosai Life Technology Co., Ltd. (CL-0233, Wuhan, China). THP-1 cells were cultured in RPMI 1640 medium (L220KJ, BasalMedia) containing 10% fetal bovine serum and 1% penicillin-streptomycin (S110JV, BasalMedia, Shanghai, China) at 37 ℃ and 5% CO_2. THP-1 cells in logarithmic growth phase were taken for subsequent experiments.

Macrophage differentiation

THP-1 cells were stimulated with 100 ng/mL PMA (IP1010, Solarbio) for 48 h to induce M0 macrophages. When the cells were transformed from suspension to a fusiform or irregular adherent state, THP-1 cells were induced to differentiate into macrophages [22]. M0 macrophages were then treated with 100 ng/mL LPS (lipopolysaccharides, LPS, IL2020, Solarbio) and 20 ng/mL IFN-γ (interferon-gamma, IFN-γ, P00028, Solarbio) for 24 h to induce the polarization of M0 macrophages to M1 macrophages [23].

Macrophage treatment and transfection

M0 macrophages in logarithmic growth phase were inoculated in 6-well cell culture plates. The cells were randomly divided into the following groups: PMA group, M1 macrophage (LPS+IFN-γ) group, miR-451 mimics (mimics) group, miR-451 inhibitor (inhibitor) group, activating transcription factor 2 (ATF2) group, LPS+IFN-γ+miR-451 mimics (LPS+IFN-γ+mimics) group, LPS+IFN-γ+ATF2 group, and the corresponding negative control group. The cells in the PMA group were routinely cultured without any operation; miR-451 mimics, miR-451 inhibitor, ATF2 and corresponding negative controls were transfected into M0 macrophages according to the instructions of Lipofectamine 3000 (L3000001, Invitrogen, Austin, TX, USA) kit. Then, the transfected or untransfected M0 macrophages were polarized to M1 macrophages by LPS and IFN-γ.

Macrophage markers were detected by flow cytometry (FCM)

Cells were collected, resuspended into a single cell suspension, stained with polarization markers (M0 macrophages: 5 μL anti-human CD68-PE (333807, Biolegend); M1 macrophages: 5 μL anti-human CD86-FITC (374203, Biolegend)), incubated in a refrigerator at 4 ℃ for 30 min, and fixed with 500 μL paraformaldehyde for flow cytometry (BD FACSCaliburTM, BD Biosciences, San Jose, CA, USA). The Cell Quest software was used for data analysis.

CCK-8 assay

After the polarization of M1 macrophages was completed, the medium was replaced with a medium containing 10% CCK-8 reagent and cultured for two hours. The $OD_{450\ nm}$ was detected by a microplate reader (MultiskanGo1510, Thermo, Massachusetts, USA), and the cell viability was calculated according to the formula.

Bioinformatics analysis

The potential target genes of miR-451 were screened by miRWalk (http://129.206.7.150/), TargetScan (https://www.targetscan.org/vert_80/), mirRDB (https://mirdb.org/), and ENCORI/starBase (https://rnasysu.com/encori/) databases. The binding site of miR-451 to ATF2 was predicted by the TargetScan database.

Dual-luciferase assay

WT-ATF2 or MUT-ATF2 targeted binding site fragments were ligated to the pmirGLO vector to construct a dual luciferase vector, and then transfected with miR-451 mimics, NC or mimics into M0 macrophages using transfection reagents. After 48 h, the cells were digested with trypsin and transferred to a 96-well microplate, and then the luciferase activity of each group was measured using the Promega dual Glo® luciferase assay system.

qRT-PCR

Total RNA was extracted from the whole blood of 20 healthy donors using TransZol Up (ET111-01-V2, TRANS, Beijing, China). Using the TransScript® Green One-Step qRT-PCR SuperMix kit (AQ211-01, TRANS), RNA was reversely transcribed into cDNA and subjected to qRT-PCR amplification. The data were quantified by the $2^{-\Delta\Delta Ct}$ method. GAPDH and U6 were used as internal references.

Primer sequences: miR-451: F: TCCGATTGAGTCATTACCAT; R: GTGCAGGGTCCGAGGT; ATF2: F: AATTGAGGAGCCTTCTGTTGTAG; R: CATCACTGGTAGTAGACTCTGGG; GAPDH: F: 5'-TGGCCTTCCGTGTTCCTAC-3'; R: 5'-GAGTTGCTGTTGAAGTCGCA-3'; U6: F: 5'-CAGGTCTCCAAGACGACATAGA-3'; R: 5'-CGCCTTTTCGATTCATGTACTGC-3'.

Western blot (WB)

Ten μM Compound C, an AMPK inhibitor, was applied at the same time as mimics transfection, followed by induction of M1 macrophage polarization. Then, the cells of each group were collected, and the total protein was extracted by adding the lysate. The protein concentrations were detected using the BCA kit (PC0020, Solarbio), and the protein was denatured at high temperature. The appropriate concentration of the gel was prepared, and electrophoresis was performed after loading the sample. The gel was then immersed in the transfer solution and transferred to the PVDF membrane at a low temperature. At the end of the incubation, the protein band was immersed in a rapid blocking solution for 30 min. CD86 (ab239075, abcam), p-mTOR (ab109268, abcam), iNOS (ab283655, abcam), TNF-α (ab215188, abcam), ATF2 (ab239361, abcam), p-AMPK (ab92701, abcam), AMPK (ab271188, abcam), mTOR (ab134903, abcam) and GAPDH (ab128915, abcam) primary antibodies were incubated overnight at 4 ℃. The corresponding secondary antibody was re-incubated, followed by PBST wash-

ing. The gel was then exposed to luminescent liquid (G2161, Servicebio) and imaged using the gel imaging system (SCG-W1000, Servicebio). Image J software was used to analyze the protein gray value. Finally, the results were saved and analyzed.

Animal experiment

Sixty-four SPF 6-week-old male C57BL/6 mice, weighing (20±2) g, were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were fed in a (22±2) ℃, 50%-60 % relative humidity, light/dark 12h/12h environment. During the feeding period, the mice were fed freely, and the experimental process was started after one week of adaptive feeding. All experiments were conducted following guidelines approved by animal protection organizations and the Jiaxing First Hospital committee.

After anesthesia with 1% pentobarbital sodium, the mice were subjected to cardiac puncture and sterile blood collection. Thirty-two mL of whole blood from 40 mice was mixed with 5.2 mL of CPDA-1 anticoagulant (14% CPDA-1 was eventually used for storage), and the whole blood of the mice was stored for 14 days. All procedures in the blood collection process were performed under strict aseptic conditions. Whole blood was mixed in a 50 mL sterile tube (Corning, New York, USA) and stored in the dark at 4 ℃.

The remaining 24 mice were used to establish a mouse model of traumatic hemorrhagic shock [24], and the stored blood samples were injected into the shocked mice through the tail vein. Shock-mice were randomly divided into four groups with six mice in each group: group 1 without exchange transfusion + equal amount of normal saline (Control), group 2 exchange transfusion + equal amount of normal saline (Model), group 3 exchange transfusion + agomir-NC (agomir-NC) and group 4 exchange transfusion + agomir-451 (agomir-451). After the exchange transfusion was completed, the mice were immediately injected with five nmol agomir-NC and agomir-451 via the tail vein. The levels of macrophage polarization markers and inflammatory cytokines were evaluated after 72 h.

After 72 hours of treatment, the mice were sacrificed, and the lymph node tissues of the mice in each group were fully ground and digested with collagenase (C8160, Solarbio) at 37 ℃ for 0.5 h. The DMEM medium containing 20% fetal bovine serum was added to the mixture to terminate the digestion entirely. After being blown, it was passed through a 70 μm sieve and centrifuged to prepare a single cell suspension. 100 μL suspension was added with CD86 antibody, incubated at 4 ℃ for 30 min in the dark, washed three times with PBS solution, and each tube was resuspended with 200 μL PBS solution and detected by flow cytometry.

ELISA

The blood sample was transferred to a sterile centrifuge tube, and the supernatant was collected after centrifugation. IL-10 (ab34843, abcam), TNF-α (ab183218, abcam), IL-6 (ab11449, abcam; sEKM-0007, Solarbio), IL-1β (ab2105, abcam; SEKM-0002, Solarbio), IL-12 (ab9992, abcam), inducible nitric oxide sythase (iNOS, ab205529, abcam), IL-18 (SEKM-0019, Solarbio), CCL22 (SEKM-0110, Solarbio) and transforming growth factor-β (TGF-β, SEKM-0035, Solarbio) levels were measured by ELISA. A total of 90 μL of sample serum and standard were added to the reaction well, and 10 μL of horseradish peroxidase-labelled antibody was added. After mixing, the reaction well was sealed with a sealing membrane and incubated for two hours in the dark. Washed with detergent three times and dried on absorbent paper. 100 μL chromogenic agent TMB solution was added, the reaction hole was sealed with a sealing film, and the reaction was incubated for 20 min. Fifty μL of stop solution was added, the OD value was immediately measured after mixing, and the corresponding concentration of the sample was determined according to the standard curve.

Statistical analysis

The Prism software (Graphpad 9.0) was used for statistical analysis, and the data with normal distribution were expressed as mean ± standard deviation. One-way ANOVA analysis was used for comparison among multiple groups. The Pearson correlation was performed for fold-change in the level of miR-451 and expression level of inflammatory factors (IL-1β, IL-6, IL-12, and TNF-α). $p<0.05$ was considered statistically significant.

RESULTS

The levels of inflammatory factors and the expression of miR-451 in whole blood storage

More and more studies have shown that RBCs will age or even undergo apoptosis after two weeks of storage due to biochemical and morphological changes. Therefore, we tested the stored whole blood samples on days 0, 21 and 35. We evaluated the fold-change in miR-451 levels and the expression levels of inflammatory factors (IL-1β, IL-6, IL-12, and TNF-α), which are typically released or overexpressed upon macrophage M1 type activation, using Pearson correlation analysis. This analysis revealed a correlation between them (Table 1). The results showed that inflammatory factors IL-1β, IL-6, IL-12 and TNF-α contents were markedly increased in the whole blood samples stored for 21 d and 35 d, and the inflammation level progressively rose with the increase of storage time (Fig.1A-D). Simultaneously, miR-451 expression was significantly decreased in whole blood samples stored for 21 d and 35 d (Fig. 1E). Notably, miR-451 had a protective effect on RBC storage damage. Compared with fresh blood samples, a large number of inflammatory factors accumulate in long-term stored blood samples. These inflammatory factors are related to M1 macrophages. Therefore, we speculate that miR-451 may be involved in improving RBC storage loss by regulating macrophage M1 polarization and mediating inflammatory response.

Table 1. Statistical analysis of correlations between levels of miR-451 and inflammatory cytokines.

		qIL-1β	qIL-6	qIL-12	qTNF-α
miR-451	Pearson Correlation	-.444*	-.519*	-.450*	-.414
	Sig. (2-tailed)	0.049	0.019	0.046	0.069
	n=20				

qIL-1β = qIL-1β Day 35/ qIL-1aDay 0,qIL-6 = IL6 Day 35/IL6 Day 0, qIL-12 = IL-12 Day35/ IL-12 Day 0, qTNF-α = qTNF-α Day 35/ qTNF-α Day 0.

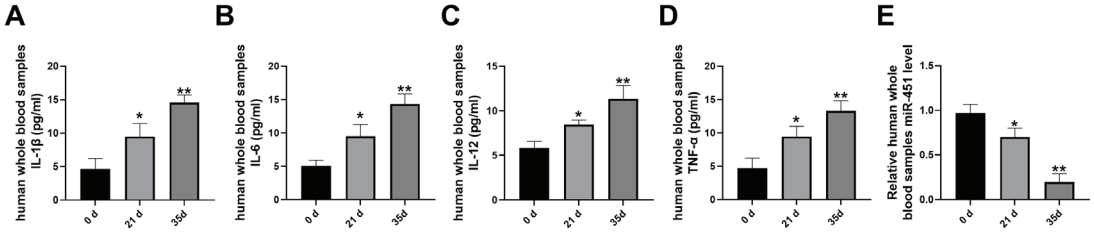

Fig. 1. Inflammatory factor contents and miR-451 expression in whole blood storage.

A-D: IL-1β, IL-6, IL-12 and TNF-α levels in human whole blood samples stored for 0 d, 21 d and 35 d were detected by ELISA, which were significantly increased at 21 d and 35 d.

E: miR-451 level in human whole blood samples stored for 0 d, 21 d and 35 d was detected by qRT-PCR. It can be seen that it was significantly reduced at 21 d and 35 d.

n=20, *$p<0.05$, **$p<0.01$ vs 0 d group vs 0 d group, one-way ANOVA was used for statistical analysis.

miR-451 attenuates macrophage M1 polarization and inhibits the release of inflammatory factors

To clarify the specific function of miR-451 on improving RBC storage damage, this study used PMA to induce THP-1 cells into M0 macrophages, and detected the expression of its marker CD68 by FCM. The results showed that PMA could significantly increase CD68-positive cells (Fig.2A-B), suggesting that M0 macrophages were successfully induced. Then, M0 macrophages were induced to polarize into M1 macrophages by LPS and IFN-γ, and the marker CD86-positive cells were significantly increased by FCM (Fig.2C-D), indicating that M1 macrophages were successfully polarized. miR-451 expression was obviously lessened in M1 macrophages (Fig.2E), suggesting that miR-451 may regulate macrophage polarization. Therefore, we next transfected miR-451 mimics into M0 macrophages; miR-451 expression was notably increased (Fig.2F),

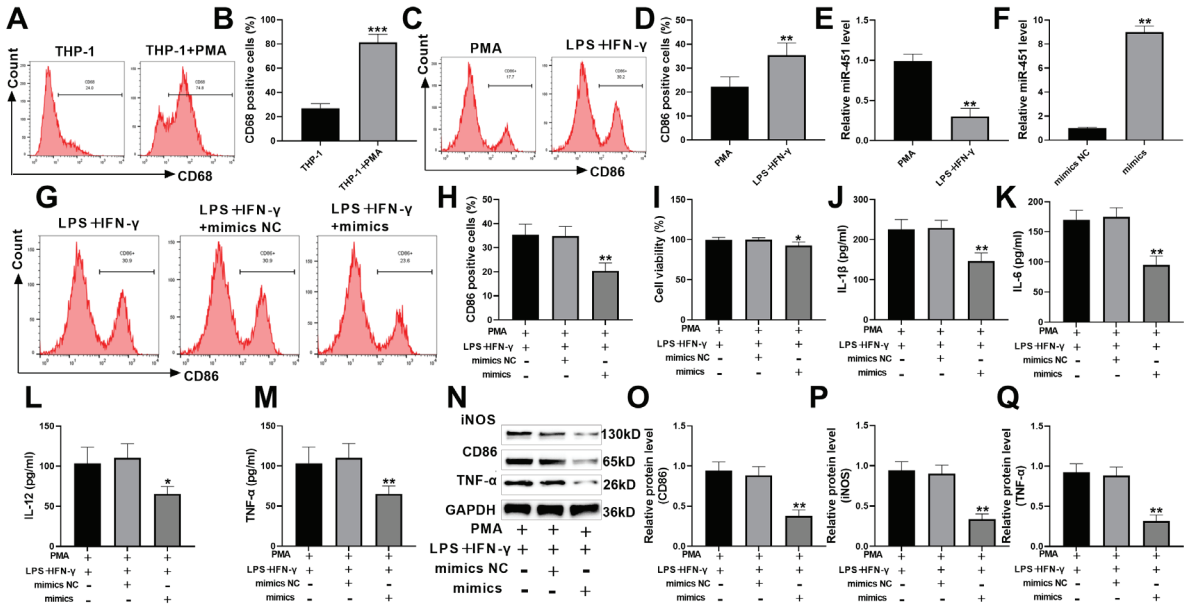

Fig. 2. miR-451 attenuates macrophage M1 polarization and inhibits the release of inflammatory factors.

A-B: M0 macrophage marker CD68 level was detected through FCM, and it was found that PMA could significantly increase CD68-positive cells.
C-D: M1 macrophage marker CD86 level was detected through FCM, and it was found that LPS and IFN-γ could significantly increase its expression.
E: miR-451 expression was discovered using qRT-PCR, and it was significantly decreased in M1 macrophages.
F: miR-451 mimics were transfected into M0 macrophages, and qRT-PCR detected the expression of miR-451, and its high expression was observed.
G-H: The transfected M0 macrophages were polarized into M1 macrophages, and FCM detected the expression of CD86. It was found that the expression of miR-451 was markedly reduced after overexpression.
I: CCK8 was used to detect the proliferation of M1 macrophages, and the cell viability was significantly reduced after miR-451 overexpression.
J-M: Proinflammatory factors IL-1β, IL-6, IL-12 and TNF-α contents in the culture medium supernatant were discovered by ELISA, and they were significantly reduced after miR-451 overexpression.
N-Q: M1 macrophage markers CD86, iNOS and TNF-α levels were detected by WB, and they were significantly reduced after miR-451 overexpression.
n=3, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs LPS+IFN-γ+mimics NC group, one-way ANOVA was used for statistical analysis.

suggesting that miR-451 was significantly overexpressed, that is, transfection was successful. After transfection, the transfected M0 macrophages were polarized into M1 macrophages. It was found that after overexpression of miR-451, the M1 macrophage marker CD86-positive cells were significantly reduced (Fig. 2G-H), and the cell viability was also significantly reduced (Fig. 2I); that is, miR-451 can inhibit macrophage M1 polarization. At the same time, ELISA also found that proinflammatory factors IL-1β, IL-6, IL-12 and TNF-α contents in the medium supernatant were significantly decreased after miR-451 overexpression (Fig. 2J-M), indicating that miR-451 inhibited the release of inflammatory factors. Finally, M1 macrophage markers CD86, iNOS, and TNF-α protein were also markedly reduced (Fig. 2N-Q). The above results indicate that overexpression of miR-451 can attenuate macrophage M1 polarization and inhibit proinflammatory factor release.

miR-451 can target ATF2 expression

The target gene of miR-451 was screened by various databases as ATF2, ENCORI/starBase targeting score≥1 (Fig. 3A). The expression of the ATF2 gene and ATF2 protein was notably decreased after overexpression of miR-451. After knocking down miR-451, ATF2 gene expression and ATF2 protein level were significantly increased (Fig. 3B-D), indicating that miR-451 can downregulate ATF2. When miR-451 was overexpressed, WT-ATF2 luciferase activity was markedly reduced, but MUT-ATF2 luciferase activity was not affected (Fig.3E), and its binding site is shown in Fig.3F. This further confirmed the binding of miR-451 to ATF2. Finally, ATF2 protein level in M1 macrophages was significantly increased (Fig. 3G-H).

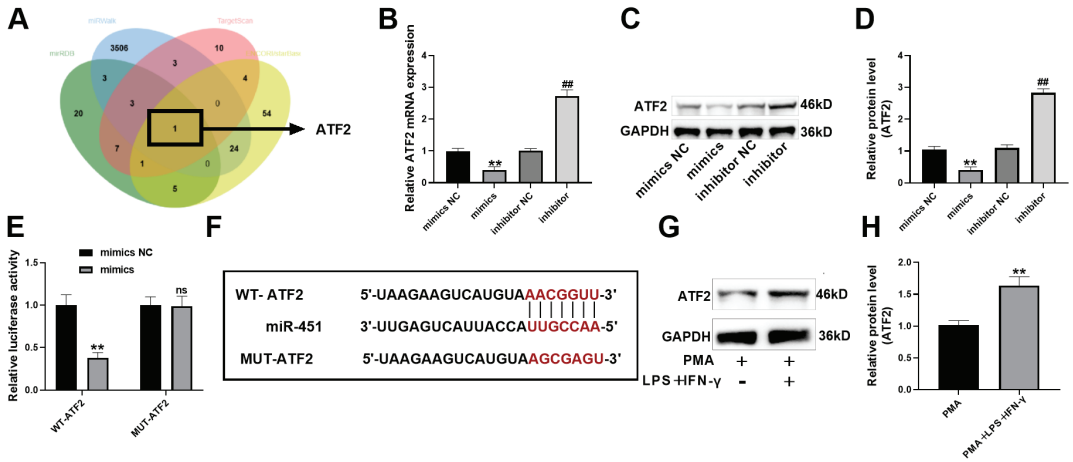

Fig. 3. miR-451 can target ATF2 expression.

A: The potential target gene ATF2 of miR-451 was screened by miRWalk, TargetScan, mirRDB and ENCORI/starBase databases.
B: The expression level of the ATF2 gene was detected by qRT-PCR, which was decreased after miR-451 overexpression and increased after miR-451 knockdown.
C-D: WB detected ATF2 protein level, which declined after miR-451 overexpression and elevated after miR-451 knockdown.
E: Dual luciferase assay showed that overexpression of miR-451 reduced WT-ATF2 luciferase activity.
F: The binding site of miR-451 and ATF2.
G-H: ATF2 level in M1 macrophages was discovered through WB, and it was significantly increased.
n=3, $^{**}p<0.01$ vs mimics NC group; $\#\#p<0.01$ vs inhibitor NC group, one-way ANOVA was used for statistical analysis.

miR-451 inhibits macrophage M1 polarization and proinflammatory cytokine release by inhibiting ATF2

Next, ATF2 was overexpressed in this study, and ATF2 gene expression and ATF2 protein level were significantly overexpressed in M0 macrophages (Fig. 4A-C), indicating that ATF2 was successfully overexpressed. Then we overexpressed miR-451 and ATF2 in M0 macrophages and polarized them into M1 macrophages using LPS and IFN-γ. Compared with miR-451 overexpression, miR-451 and ATF2 overexpression increased CD86-positive cells (Fig. 4D-E), and the protein levels of CD86, iNOS and TNF-α were also notably raised (Fig. 4F-I). IL-1β, IL-6, IL-12 and TNF-α contents in the supernatant were significantly increased (Fig. 4J-M). ATF2 mitigated the inhibitory effect of miR-451 on macrophage M1 polarization. Combined with bioinformatics and miR-451 overexpression experiments, it was demonstrated that miR-451 could target ATF2 to attenuate macrophage M1 polarization and inflammation.

miR-451 inhibits macrophage M1 polarization by inhibiting ATF2 and regulating AMPK/mTOR signaling pathway

miR-451 can regulate cell function through the AMPK/mTOR signalling path-

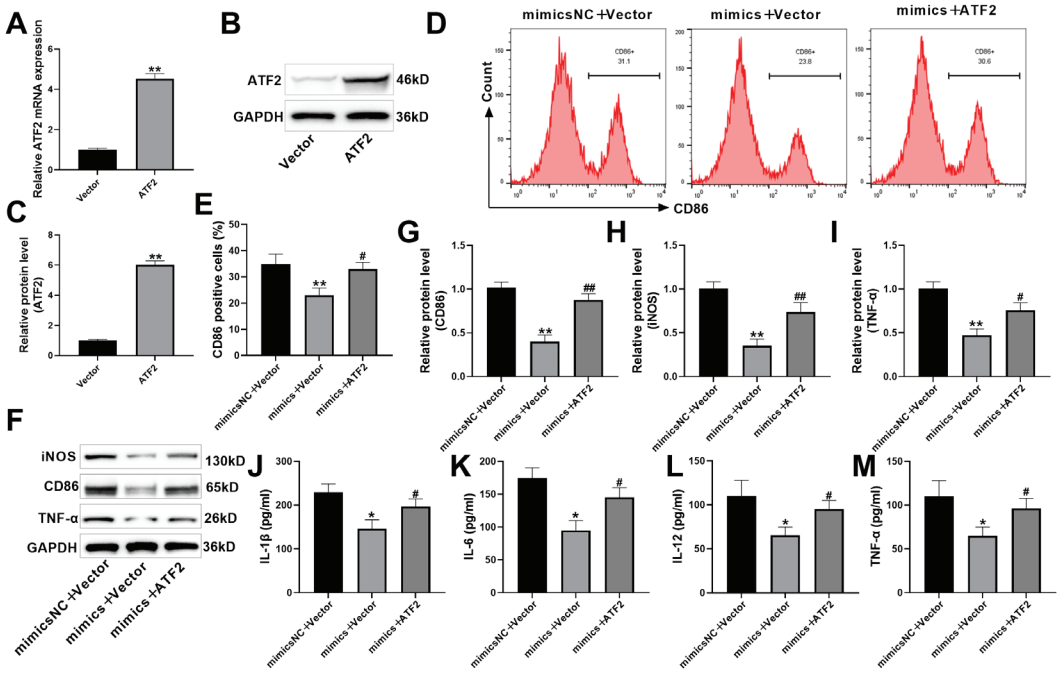

Fig. 4. miR-451 inhibits macrophage M1 polarization and proinflammatory cytokine release by inhibiting ATF2.

A-C: The overexpression level of ATF2 was detected by qRT-PCR and WB, and the gene expression and protein level of ATF2 were significantly increased.
D-E: The transfected M0 macrophages were polarized into M1 macrophages, and the expression of CD86 was detected by FCM, which was significantly increased upon ATF2 overexpression.
F-I: CD86, iNOS and TNF-α levels were discovered through WB, and they were significantly increased after ATF2 overexpression.
J-M: IL-1β, IL-6, IL-12 and TNF-α contents in the medium supernatant were discovered by ELISA, and they were significantly increased after ATF2 overexpression.
n=3, *$p<0.05$, **$p<0.01$ vs mimics NC+Vector group; #$p<0.05$, ##$p < 0.01$ vs mimics+Vector group, one-way ANOVA was used for statistical analysis.

way. In this study, AMPK inhibitor compound C was used for intervention experiments. p-AMPK protein levels were elevated, and p-mTOR protein expression declined when miR-451 was overexpressed. On this basis, p-AMPK expression lessened and p-mTOR expression increased after overexpression of ATF2 and application of compound C (Fig.5A-C), indicating that miR-451 regulates AMPK/mTOR signalling pathway by inhibiting ATF2. At the same time, CD86, iNOS and TNF-α levels were markedly raised after compound C intervention (Fig. 5D-G), indicating that inhibition of the AMPK/mTOR pathway could promote macrophage M1 polarization. In conclusion, miR-451 attenuates macrophage M1 polarization by targeting ATF2 to regulate the AMPK/mTOR axis.

miR-451 regulates transfusion immunity in mice infused with traumatic shock

Finally, this study established a mouse model of traumatic hemorrhagic shock, performed blood exchange operations, and injected agomir-451 intravenously. In Model mice, the number of CD86-positive cells increased, suggesting that macrophage M1 polarization occurred. After miR-451 overexpression, CD86-positive cells decreased (Fig. 6A-B), indicating that miR-451 can also inhibit macrophage M1 polarization *in vivo*. At the same time, it was also found in the blood that the contents of TNF-α, IL-6, iNOS, IL-18, IL-1β, CCL22, and TGF-β increased in Model mice, and significantly decreased after miR-451 overexpression (Fig. 6C-I), indicating that miR-451 can also inhibit inflammation *in vivo*. In summary, miR-451 can regulate

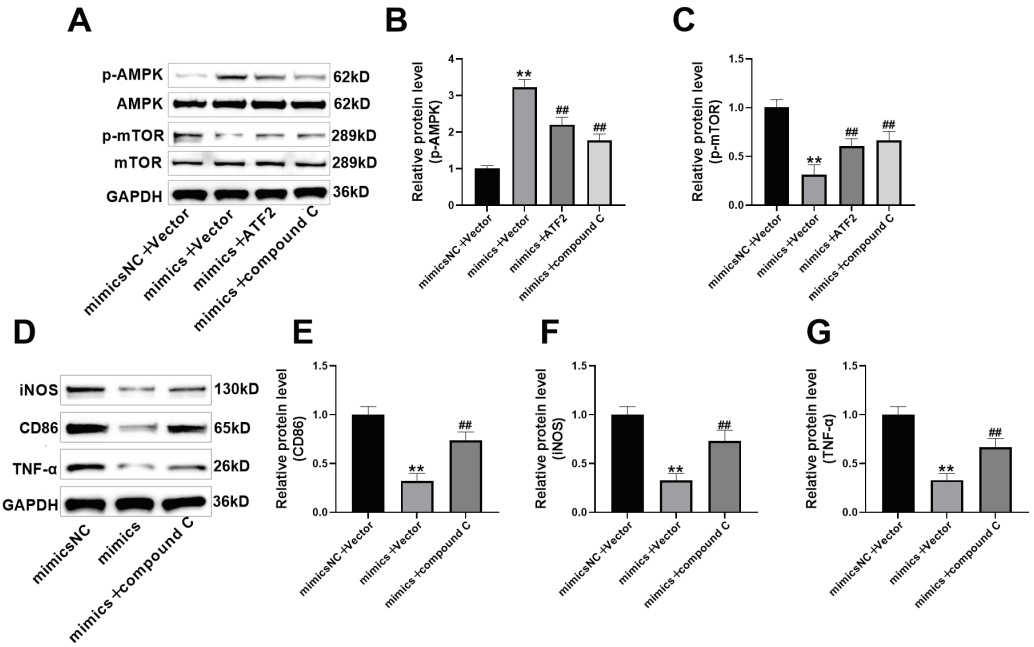

Fig. 5. miR-451 inhibits macrophage M1 polarization by inhibiting ATF2 and regulating the AMPK/mTOR signalling pathway.

A-C: WB was used to detect the expression of AMPK/mTOR pathway proteins. It was found that the level of p-AMPK increased and p-mTOR protein decreased when miR-451 was overexpressed. On this basis, ATF2 overexpression and application of compound C were significantly reversed.
D-G: CD86, iNOS and TNF-α levels were discovered through WB, and they were significantly increased after compound C intervention.
n=3, $^{**}p<0.01$ vs mimics NC+Vector group; $\#\#p<0.01$ vs mimics+Vector group, one-way ANOVA was used for statistical analysis.

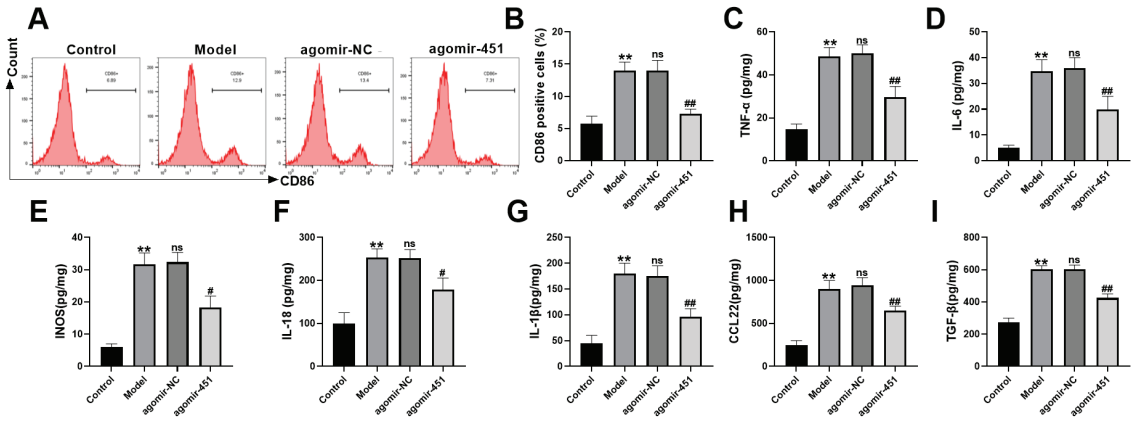

Fig. 6. miR-451 infusion regulates transfusion immunity in mice with traumatic shock.

A-C: The expression of CD86 was detected by FCM, which was increased in Model mice and decreased after miR-451 overexpression.
D-G: The blood inflammatory cytokines TNF-α, IL-6, iNOS, IL-18, IL-1β, CCL22 and TGF-β expressions were detected by ELISA. It can be seen that they increased in Model mice and decreased after miR-451 overexpression.
n=6, $^{**}p<0.01$ vs Control group; $\#\#p<0.01$ vs Model group, one-way ANOVA was used for statistical analysis.

blood transfusion immunity in mice infused with stored RBCs by inhibiting macrophage M1 polarization and inflammation.

DISCUSSION

In recent years, with the continuous improvement of medical level and surgical volume, the demand for clinical blood is increasing. At present, the primary source of blood transfusion is still allogeneic stored RBC. In recent years, RBC storage damage has been the focus of blood transfusion studies. RBC is one of the most widely used blood components in clinical practice. However, RBC in the middle and late stages of infusion storage is not without risk [25]. Because RBC is stored at sub-physiological pH and temperature, a series of metabolic, oxidative and physiological changes occur in RBC, which are called RBC storage lesions. Many of these changes increase with time, affecting the overall quality standards, functional integrity and *in vivo* survival of RBC infusion. Some changes in the early storage period (about 14 days) are reversible, but with storage time extension, the damages are irreversible [26].

Studies have confirmed that after RBC is isolated, the low-temperature storage environment will lead to electrolyte imbalance, metabolite accumulation, oxidative stress, membrane damage, and ultimately lead to storage damage [27]. These changes damage RBC metabolism, function, and viability. However, the mechanism of RBC storage damage is still unclear. The gene chip study revealed that 109 miRNAs increased and 102 genes decreased during storage, when RBCs were stored for 20 days compared with the fresh group. Sarachana et al. [29] found four microRNAs related to RBC storage damage. RT-PCR analysis showed that these four miRNAs were differentially expressed on day 14 and day 28. Bioinformatics analysis identified the potential targets and biological functions of these miRNAs. Overexpression of miR-196a in human RBC cell lines confirmed its protective effect on cell death and ATP loss [29]. It has been found that miR-451 is the most expressed miRNA in mature RBCs. At the same time, some studies have

reported that RBC microparticles can mediate the transmission of miR-451 to endothelial cells and affect the results after RBC transfusion [30], suggesting that miR-451 may mediate RBC storage damage and may also affect blood transfusion receptors through microparticles, exosomes and other ways to cause adverse transfusion events.

Studies have shown that the old RBCs with a long storage period (≥ 2 weeks) release the most abundant heme content and the most obvious damage-related molecular pattern. Heme can amplify the LSP-induced inflammatory response, and a large number of old RBCs are infused into special patients, such as those with traumatic hemorrhagic shock and sepsis. The free heme will exceed the body's metabolic capacity and promote the body's inflammatory damage. It was found that IL-1β, IL-6, IL-12 and TNF-α were highly expressed in long-term stored whole blood [31]. Infusion of suspended, less white RBCs can cause an increase in inflammatory factors and initiate neutrophil activity.[32] Therefore, it can be speculated that infusion of long-term stored RBCs may cause explosive release of inflammatory factors, and ultimately cause human anti-RBC allogeneic immune response.

In addition to the role of oxygen supplementation, RBCs also have apparent immunomodulatory effects [33, 34]. Studies have found that both RBC metabolites and surface molecules have immunomodulatory effects. For example, the metabolite heme molecules can stimulate macrophages to induce inflammatory responses and interfere with RBC function [35]. The CD47 molecule on the surface of red blood cells acts as a 'do not eat me' signal, which is directly involved in the recognition and phagocytosis of red blood cells by macrophages. [36]. RBC immune factor is one of the critical reasons for ineffective infusion. At this stage, storage of RBC can affect the inflammatory expression of macrophages. The immune effect of RBC has attracted attention in recent years, and RBC can produce a variety of immunomodulatory substances during storage [26]. Its molecular mechanism is complex and has not been fully elucidated.

Macrophages are key cell populations associated with the body's innate immune response, which not only affect the body's immune function, but also affect the metabolism of RBC [37]. Macrophages can differentiate into M1 and M2 types, which are two types of cell populations with different functions. This process is called macrophage polarization [38, 39]. M1 macrophages (mainly expressing cell membrane proteins such as CD86, CD80 and MHC-II) secrete proinflammatory response factors, while M2 macrophages (mainly expressing cell membrane proteins such as CD206 and CD103) secrete anti-inflammatory cytokines to exert anti-inflammatory effects [40]. M1 macrophages can increase IL-1β and TNF-α contents, aggravate host cell damage and hinder tissue repair. In the inflammatory environment, red blood cell distribution width (RDW) increased compared with a typical environment, and inflammation had a particular effect on RBCs, maintaining the normal morphology [41]. In summary, the polarization of macrophages presents different immune functions, and M1 and M2 types may affect the survival and proliferation of RBC.

This study found that IL-1β, IL-6, IL-12 and TNF-α contents were significantly elevated in whole blood samples stored for 21 and 35 days. However, the miR-451 level was significantly decreased. The Pearson correlation analysis showed that there was a correlation between miR-451 and M1 macrophages, suggesting that miR-451 may be involved in RBC storage damage by regulating macrophage polarization-mediated inflammatory response. To this end, this study first used PMA to induce THP-1 cells into M0 macrophages, and then polarized M0 macrophages into M1 macrophages by LPS and IFN-γ, and the miR-451 expression was significantly reduced in M1 macrophages. Next, this study transfected miR-451 mimics into M0 macrophages and then polarized them

into M1 macrophages. It was found that the activity of M1 macrophages was declined, and the markers CD86, iNOS expression and inflammatory factors IL-1β, IL-6, IL-12, and TNF-α contents were significantly reduced. In summary, the overexpression of miR-451 can attenuate macrophage M1 polarization and restrain the release of proinflammatory factors.

In this study, ATF2 was identified as a potential target gene of miR-451. Overexpression of miR-451 reduced ATF2 levels, while knockdown of miR-451 increased ATF2 levels. The two have binding sites, indicating that miR-451 can target and downregulate ATF2. When harmful substances stimulate macrophages, G protein-coupled receptors and non-G protein-coupled receptors are activated, leading to increased phosphorylation of MAPK protein 42. This MAPK then enters the nucleus and activates the nuclear transcription factor ATF2, which promotes the secretion and release of proinflammatory cytokines IL-6, IL-1β, and TNF-α 43. Simultaneously, this study also found that the level of ATF2 protein in M1 macrophages was notably increased. After overexpressing ATF2 in M0 macrophages, they were polarized into M1 macrophages. Compared to the overexpression of miR-451 alone, ATF2 overexpression increased the levels of M1 macrophage markers CD86, iNOS, and inflammatory factors IL-1β, IL-6, IL-12, and TNF-α. Overall, miR-451 targets ATF2 to reduce macrophage M1 polarization and inflammation.

As a cell energy sensor, AMPK is responsible for regulating cell energy and material metabolism. mTOR is mainly involved in cell growth, proliferation and metabolism. AMPK is also an important upstream regulator of mTOR [44]. AMPK is an enzyme complex that is crucial for macrophage polarization and inflammation. Activation of the AMPK signalling pathway can not only reduce inflammatory response in adipose tissue of obese mice, but also inhibits macrophage M1 polarization [45,46]. It is known that protein kinase B and the downstream signalling molecule mTOR play the key roles in regulating macrophage polarization. mTOR is activated by phosphorylation and exists in the form of mTOR complex mTORC1 and mTORC2 in cells. Inhibition of mTOR causes macrophages to polarize to the proinflammatory phenotype M1 [47]. Activation of mTORC1 may lead to a decrease in the anti-inflammatory phenotype M2 polarization [48]. The M1 polarization of alveolar macrophages is considered to be essential for the pathogenesis of transfusion-related acute lung injury [49]. Macrophage cells undergo functional changes and severe reactions upon ingesting transfusion RBC microparticles [50], suggesting that miR-451 may regulate macrophage polarization by modulating the mTOR signalling pathway, thereby mediating inflammatory responses and contributing to the promotion of post-transfusion complications. This study showed that p-AMPK protein levels rose and p-mTOR protein expression lowered when miR-451 was overexpressed. When ATF2 was overexpressed and the AMPK inhibitor compound C was applied, the protein level of the AMPK/mTOR pathway was significantly reversed. In addition, this study also found that M1 polarization of macrophages increased significantly after compound C intervention. Overall, miR-451 could target ATF2 to regulate the AMPK/mTOR axis to attenuate macrophage M1 polarization and participate in the immune response of stored RBCs.

Clinical practice shows that both immune-related and non-immune-related factors during RBC transfusion can cause adverse reactions to blood transfusion. These adverse reactions are closely related to RBC transfusion. RBC storage damage is closely associated with the incidence of adverse consequences of blood transfusion. Infusion of suspended RBC with 'storage damage' will increase the incidence of adverse transfusion reactions and mortality [51]. Infusion of stored RBCs not only damages the oxygenation capacity of tissues, but also the RBCs entering the body react with macrophages

to become injury or risk-related molecules, which further activate the innate immune response and lead to the damage of corresponding normal tissues or organs [52]. Finally, this study found that M1 macrophages and inflammatory factors were significantly increased in mice undergoing infusion, while miR-451 overexpression reduced M1 macrophages and inflammatory factor levels.

In summary, the data indicated that the inhibition of ATF2 by targeting miR-451 regulates the AMPK/mTOR pathway, which suppresses the polarization of macrophages to the M1 phenotype and reduces the inflammatory response. This process, in turn, diminishes the damage to red blood cells (RBCs) during storage and lessens the immune response triggered by the infusion of stored RBCs (see Fig. 7). This study offers a new approach and methodology for decreasing RBC storage damage. However, research on RBCs should extend beyond their physiological roles; they should not be viewed solely as cells responsible for supplying oxygen. The immune regulation of stored RBCs may be a key factor in adverse infusion reactions, and changes in macrophage function are a significant aspect of this immune regulation. Focusing on the immune function of macrophages is a vital direction for studying the immune mechanisms underlying adverse infusion reactions. Nevertheless, considering the complexity and diversity of immune responses following infusion, our research team will further design and explore relevant research paths.

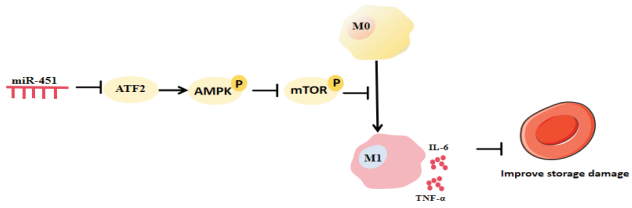

Fig. 7. miR-451-targeted inhibition of ATF2 to regulate AMPK/mTOR pathway, inhibit macrophage M1 polarization, and improve RBC storage damage and transfusion immunity.

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Consent to publish

The manuscript has neither been previously published nor is it under consideration by any other journal. The authors have all approved the content of the paper.

Consent to participate

We secured a signed informed consent form from every participant.

Ethic approval

The Jiaxing First Hospital Committee approved this study.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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Author contribution

XW: Edited and refined the manuscript with a focus on critical intellectual contributions. XC, HZ: Developed and planned the

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Vitamin D attenuates epithelial-mesenchymal transition of renal tubular epithelial cells in infant rats with hypothyroidism via the Traf6/TAK1 signalling pathway.

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Keywords: vitamin D; hypothyroidism; kidney injuries; epithelial-mesenchymal transition; TRAF6 protein; MAP3K7 protein (TAK1); signal transduction.

Abstract. In the hypothyroidism(HT) state, renal hemodynamics is disordered, oxidative stress is intensified, and inflammatory factors are activated. Vitamin D (VD) not only regulates calcium and phosphorus metabolism, but its active form (1,25-dihydroxyvitamin D) could also exert anti-inflammatory and anti-fibrotic effects by binding with the vitamin D receptor (VDR) widely distributed in the kidney. This study aimed to elucidate the impact of VD on the epithelial-mesenchymal transition (EMT) of renal tubular epithelial cells in HT-induced renal injury in young rats, as well as its regulatory mechanism involving the tumor necrosis factor receptor-associated factor 6 (Traf6)/transforming growth factor-β activated kinase 1 (TAK1) pathway. An HT model in young rats was established via propylthiouracil (PTU) gavage, and a functional rescue experiment was conducted by overexpressing TAK1 (pcDNA3.1-TAK1). The animals were divided into five groups: normal, HT, low-dose VD (HT+VD-L), high-dose VD (HT+VD-H), and HT+VD-H+pcDNA3.1-TAK1 (HT+VD-H+pc). Each group consisted of 10 rats. Serum creatinine (Scr) and blood urea nitrogen (BUN) levels were measured using an automatic biochemical analyzer. Renal apoptosis (TUNEL), TGF-β1/α-SMA/E-cadherin (immunohistochemistry), and Bcl-2/Bax/Traf6/TAK1/p-TAK1 (Western blot) expressions were assessed in renal tissue. VD significantly reduced Scr and BUN levels in the serum of HT young rats, downregulated renal tissue apoptosis, decreased TGF-β1 and α-SMA expressions, and upregulated E-cadherin expression. Additionally, VD inhibited Traf6, p-TAK1, and Bax expressions while increasing Bcl-2 expression. All differences were statistically significant.

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Vitamina D atenúa la transformación mesenquímica-epitelial de células epiteliales tubulares renales a través de la vía de señalización Traf6/TAK1 en ratas bebé con hipotiroidismo.

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Palabras clave: vitamina D; hipotiroidismo; daño renal; transición epitelio-mesénquima; proteína TRAF6; proteína MAP3K7 (TAK1); transducción de señales.

Resumen:El hipotiroidismo (HT) altera la hemodinámica renal, el estrés oxidativo y la inflamación. La vitamina D (VD) regula calcio/fósforo; su forma activa ejerce efectos antiinflamatorios/antifibróticos vía el receptor VD renal. Este estudio se centra en observar y aclarar el efecto de la VD sobre la transición epitelial-mesenquimal (EMT) de las células epiteliales tubulares renales en la lesión renal inducida por el HT en ratas jóvenes, así como su mecanismo regulador que involucra la vía del receptor del factor de necrosis del tumor 6 (traf 6) / y del factor de crecimiento transformante β activado por la quinasa 1(TAK1). El modelo de HT se estableció en ratas jóvenes mediante el método de alimentación por sonda de propil-tiouracilo (PTU) y se realizó un experimento de rescate funcional mediante la sobreexpresión de TAK1 (pcDNA3.1-TAK1). Los animales fueron divididos en cinco grupos: normal, HT, VD de baja dosis (HT+VD-L), VD de alta dosis (HT+VD-H), HT+VD-H + PC DNA 3.1-tak 1 (HT+VD-H + PC). Cada grupo estuvo compuesto por 10 ratas. Se determinaron los niveles de creatinina sérica (Scr) y nitrógeno de urea en sangre (BUN) con un analizador bioquímico automático. Se evaluó la expresión de la apoptosis renal (TUNEL), TGF-β1/α-SMA/E-Calcinina (immunohistoquímica) y Bcl-2/Bax/traf 6/tak 1/p-tak 1 (Western blot) en el tejido renal. VD redujo significativamente los niveles de SCR y BUN en suero de ratones de HT, redujo la apoptosis en tejido renal, redujo la expresión de TGF-β1 y α-SMA y aumentó la expresión de E-calciferina. Además, la VD inhibe la expresión de Traf6, p-TAK1 y Bax, mientras que aumenta la expresión de Bcl-2.

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INTRODUCTION

Hypothyroidism (HT) is a common disease of the endocrine system, which can occur at all ages. It occurs in fetuses, newborns and infants, which could directly affect the development of children's nervous system and skeletal system, directly lead to short stature or permanent mental retardation, and bring unpredictable harm to patients' families [1]. Studies have shown that HT directly causes changes in renal hemodynamics and glomerular filtration performance[2,3], which directly leads to certain renal interstitial fibrosis. Consequently HT patients are often accompanied by certain renal dysfunction, but the pathological mechanism of HT renal injury has not been fully clarified, and there is still a lack of specific drugs in clinic, especially for infants with HT-induced renal injury. Studies have shown that abnormal apoptosis in renal tissue induced by inflammatory stress plays an important role in the progression of the disease. Adan et al. [4]

showed that the expression of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) is increased in the serum of patients with insufficient thyroxine synthesis, and it was correlated with the expression of apoptosis-inhibiting protein B cell lymphoma-2 (Bcl-2). It was positively correlated with the expression of Bcl-2-related X protein (Bax). Narilla et al. [5], showed that the levels of translation and transcription of transforming growth factor-β 1 (TGF-β1) and α-smooth muscle actin (α-SMA) in the serum of patients with hypothyroidism were significantly increased. However, the transcription and translation levels of E-cadherin decreased significantly, and the patients exhibited specific pathological changes indicative of epithelial-mesenchymal transition (EMT) in renal tubules. Tumor necrosis factor receptor-associated factor 6 (Traf6)/ transforming growth factor β-activated kinase 1 (TAK1) signal is an endogenous pathway closely related to inflammatory stress and cell fibrosis. Wang et al. [6] demonstrated that inhibiting the TRAF6/TAK1 pathway and reducing the phosphorylated TAK1 (p-TAK1) expression in the HK-2 renal tubular fibrosis model induced by high glucose significantly suppressed myofibroblast marker α-SMA expression in HK-2 cells, thereby blocking cell fibrosis progression. Many clinical data and basic experimental data at home and abroad shows that vitamin D (VD) is not only a simple fat-soluble vitamin, but also a steroid hormone closely related to physiological processes such as immune regulation, cell stress and apoptosis. Therefore, in this study, the HT-model of young rats was established by intragastric administration of propylthiouracil (PTU). Based on the TRAF6/TAK1 signal, a function rescue experiment was conducted by overexpressing TAK1 (pc DNA3.1-TAK1). The effect of VD on the EMT of renal tubular epithelial cells in young rats with HT is discussed, aiming to provide a reliable direction for the clinical treatment of the disease.

MATERIALS AND METHODS

Test animals, main reagents and instruments

Fifty 3-week-old male SD rats with SPF cleanliness, weighing 45g～55g, were used in the experiment after being fed adaptively by the specialized personnel in our hospital at a temperature of 20℃～25℃ and a humidity of 55～60%)% for 12h. Experimental reagent vitamin D drops (Star Shark) (VD, 400U*36 pills, Star shark pharmaceutical (Xiamen) Co., Ltd., Sinopharm zhunzi H35021450); PTU (purity ≥99.9%) was purchased from sigma company in USA, and overexpression TAK1(pc DNA3.1-TAK1) and negative control pc DNA3.1-TAK1-NC were purchased from anbote genetic engineering technology Co., Ltd, Beijing. Other reagents and equipment were: Phosphate buffer solution (PBS), formaldehyde, xylene, neutral resin (Beijing Suolaibao Biotechnology Co., Ltd.), rabbit anti-rat Traf6, TAK1 and p-TAK1, Bcl-2, Bax, glyceraldehyde triphosphate dehydrogenase (GAPDH) monoclonal antibody (Shanghai Biyuntian Biotechnology Co., Ltd.), Immunofluorescence, immunohistochemistry and hematoxylin and eosin (HE) staining kit (Shanghai Wohong Biotechnology Co., Ltd.), Protein western blot kit, deoxynucleotide terminal-mediated nick end labeling (TUNEL) kit (Nanjing Senbeijia Biotechnology Co., Ltd.), enzyme-linked immunosorbent assay kit (Nanjing Jiancheng Biotechnology Co., Ltd.). Tissue scissors, hemostatic forceps and other surgical instruments (Jinhua Yidi Medical Equipment Co., Ltd., Jinhua City, Zhejiang Province), Image Quant LAS4010 gel imaging system (GE Company, USA), CFX96 real-time fluorescent quantitative polymerase chain reaction (quantitative real-time polymerase chain reaction, QRT-PCR) amplifier (Bio-Rad Company, USA), Leica DMI6000B microscope (Leica Company, Germany), Z-5000 spectrophotometer (Hitachi Company, Japan), CK-150 high-speed freezing centrifuge (Sigma Company, USA).

Replication of the HT rat model

According to the method introduced by Santos et al.[7], a propylthiouracil (PTU) solution was prepared with normal saline at a concentration of 0.5% (5 mg/mL). Rats were treated by gavage with 10 mL/kg PTU solution every day, and at the same time, their weigh was recorded, and the dosage of PTU was adjusted every week according to their weight. After four weeks of continuous gavage, 2mL of tail vein blood of rats in each group was taken to detect thyroid stimulating hormone (thyroid-stimulating hormone, TSH) and total thyroxine (TT4), compared with the normal group, the serum TSH level of HT rats increased significantly, while TT4 level decreased significantly, which indicated that the model was successful.

Packet processing

After adaptive feeding, rats were randomly divided into a normal group, HT group, VD low-dose (HT+VD-L) group, VD high-dose (HT+VD-H) group and HT+VD-H+PCDNA3.1-Tak1 (HT+VD-H+PC) group, with 10 rats in each group. Rats in the HT+VD-L group were injected with 5×10^9 pfu/mL of pc DNA3.1-TAK1-NC via tail vein every day, and treated with 0.25 mg/kg of VD by gavage. Rats in the HT+VD-H group were injected with 5×10^9 pfu/mL of pc DNA3.1-TAK1-NC via the tail vein every day, and perfused with 1 mg/kg of VD. Rats in the HT+VD-H+pc group were injected with 5×10^9 pfu/mL of pc DNA3.1-TAK1 by the tail vein every day, and treated with 1 mg/kg of VD by gavage. Rats in the normal group were injected with 5×10^9 pfu/mL of pc DNA3.1-TAK1-NC by the tail vein every day, and treated with the same dose of normal saline by gavage for four weeks. Rats in the HT, HT+VD-L, HT+VD-H and HT+VD-H+pc groups still need to be treated with PTU to maintain the decline of thyroid function in experimental animals. During the experiment, rats were raised in a single cage, with access to drinking water and food.

Sample collection

After the last drug administration, 24-hour urine samples were collected from rats in each group to detect the levels of 24-hour urinary albumin (UAlb) in the offspring rats. Subsequently, the rats were anesthetized with an intraperitoneal injection of 30 mg/kg pentobarbital sodium. Then, 5 mL of blood was collected from the heart and placed in a benchtop refrigerated centrifuge for 15 minutes to obtain serum for testing. The rats were euthanized by cervical dislocation. Under a microscope and on a sterile operating table, the kidney tissues of the offspring rats were dissected.

Thyroid and renal function of rats in each group

According to the requirements of the ELISA kit, corresponding solutions were added to the standard wells and sample wells, and blank wells were set up. After antibody coating, plate washing, and reaction termination, the OD450 values of each well were measured using a multifunctional microplate reader. A standard curve was plotted, and the concentrations of TSH and TT4 in animal serum were calculated. At the same time, the levels of UAlb, serum creatinine (Scr), and blood urea nitrogen (BUN) in rats from each group were detected using an automatic biochemical analyzer.

Apoptosis in renal tissue of rats in each group

Partial renal tissue from rats in each group was taken and fixed, sliced, and washed according to the preliminary requirements of the TUNEL kit. TUNEL working solution was added, and the samples were incubated at 37°C in the dark. After dehydration and mounting, microscopic examination was performed. The apoptosis rate in renal tissue was statistically analyzed using the Image-Pro 6.2 software.

PCR detection of gene expression in renal tissue of rats in each group

Partial renal tissue samples from rats were collected, and an appropriate amount of Trizol lysis solution was added. Total RNA was extracted according to the requirements of the Trizol kit. After determining its concentration using a Z-5000 spectrophotometer, cDNA was synthesized using the Prime Script™ kit. qRT-PCR amplification was performed under the following conditions: initial denaturation at 95°C for 5 seconds, denaturation at 95°C for 5 seconds, annealing at 60°C for 60 seconds, for 40 cycles. The primer sequences are shown in Table 1. The relative expression levels of each target gene were represented by $2^{-\Delta\Delta CT}$.

Immunofluorescence detection of TNF-α and IL-6 expression in renal tissue of rats in each group

Partial renal tissue samples from rats were collected and subjected to fixation, embedding, slicing, microwave repair, and incubation in 3% hydrogen peroxide. After washing with PBS, the samples were blocked with goat serum and then incubated with primary antibodies against TNF-α and IL-6 (diluted 1:500) at 4°C overnight in the dark. The next day, fluorescent secondary antibodies were added, and the samples were incubated in the dark. Observation was performed under a microscope.

Immunohistochemical experiment to detect the expression of E-cadherin, α-SMA, and TGF-β1 in renal tissue

Partial renal tissue samples from rats were fixed, embedded, and sliced, followed by immunohistochemical staining according to the kit instructions. Primary antibodies against E-cadherin, α-SMA, and TGF-β1 (all diluted 1:500) and secondary antibodies (diluted 1:1500) were added. Observation was performed under a microscope, and statistical analysis was conducted using the Image J image processing software.

Expression levels of Bcl-2, Bax, Traf6, TAK1, and p-TAK1 in renal tissue of rats in each group

renal tissue from rats in each group was placed in lysis buffer at 4°C for 30 minutes, followed by centrifugation and dilution of the supernatant. Total protein was extracted from the samples using RIPA lysate. Fifty micrograms of protein samples were loaded for electrophoresis, followed by membrane transfer and blocking. Primary antibodies against Bcl-2, Bax, Traf6, TAK1, and p-TAK1 (all diluted 1:1000) and secondary antibodies (diluted 1:5000) were added, and the samples were incubated at room temperature. After color development for 30 minutes, GAPDH was used as a reference to analyze the grayscale values of the target proteins.

Statistical analysis

The SPSS19.0 software (International Business Machines Corporation, New York, USA) and Graphpad5.01 software (GraphPad Software Inc., San Diego, CA, USA) were used for statistical analysis of data, and the data were expressed as mean±SD. The t-test was conducted to compare the two groups, and the comparison between multiple groups was conducted by one-way analysis of variance, with $p<0.05$ indicating statistical significance.

RESULTS

Comparison of thyroid function of rats in each group

The results of the enzyme-linked adsorption kit showed that compared with normal rats, the serum TT4 levels in the HT, HT+VD-L, HT+VD-H and HT+VD-H+pc groups were reduced significantly, while the TSH level increased significantly. Compared with the HT group, the serum TT4 levels in the HT+VD-L group, HT+VD-H group, and HT+VD-H+pc group increased significantly, while the TSH level decreased significantly.

Compared with the HT+VD-L group, the level of TT4 in the serum of the HT+VD-H group was significantly higher, while the level of TSH was significantly lower. Compared with the HT+VD-H group, the level of TT4 in the serum of the HT+VD-H+pc group decreased significantly, and the level of TSH increased significantly, with statistical significance (all $p<0.05$) (Fig. 1).

Changes in renal function in each group of rats

The results showed that, compared with the normal group, the contents of UAlb, Scr, and BUN in serum of rats in the HT, HT+VD-L, HT+VD-H, and HT+VD-H+pc groups were significantly higher. In the HT+VD-L group, HT+VD-H group and HT+VD-H+pc group, the contents of UAlb, Scr and BUN in serum decreased significantly. Compared with the HT+VD-L group, the contents of UAlb, Scr and BUN in the serum of the HT+VD-H group decreased significantly (Fig. 2).

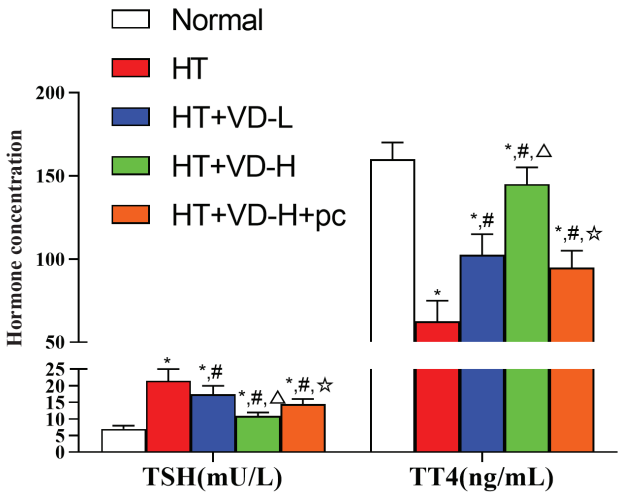

Fig. 1. Changes of Thyroid stimulating hormone (TSH) and Total thyroxine (TT4) in rats in each group. Note: One-way ANOVA. Compared with the Normal group, *: $p<0.05$ compared with HT group; #:$p<0.05$ compared with HT+VD-L group; Δ:$p<0.05$ compared with HT+VD-H group; ☆: $p<0.05$).

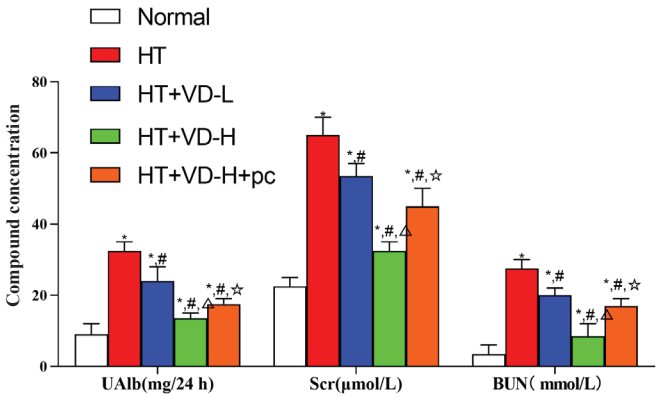

Fig. 2. Changes of 24-hour urinary albumin (UAlb), Serum creatinine (Scr) and Blood urea nitrogen (BUN) in rats of each group (Note: One-way ANOVA. Compared with the Normal group, *:$p<0.05$ compared with the HT group; #:$p<0.05$ compared with HT+VD-L group; Δ:$p<0.05$ compared with HT+VD-H group; ☆:$p<0.05$)

Apoptosis in the renal tissue of rats in each group

TUNEL staining results showed that compared with the normal group, the apoptosis rate in the kidney tissue of rats in the HT, HT+VD-L, HT+VD-H and HT+VD-H+pc groups was significantly higher. Compared with the HT group, the cells in the kidney tissue of rats in HT+VD-L, HT+VD-H and the HT+VD-H+pc groups were significantly higher. The apoptosis rate in the kidney tissue of the HT+VD-H group decreased significantly, and compared with the HT+VD-H group, the apoptosis rate in the kidney tissue of the HT+VD-H+pc group increased significantly, with statistical significance (all $p<0.05$) (Fig. 3).

Expression of related genes in the renal tissue of rats in each group

The results of PCR showed that compared with the normal group, the mRNA expressions of TNF-α, IL-6, α-SMA, TGF-β1, Traf6 and TAK1 in the renal tissues of the HT, HT+VD-L, HT+VD-H and HT+VD-H+pc groups were significantly increased, while the mRNA expression of E-cadherin was significantly decreased.

Compared with the HT group, the mRNA expressions of TNF-α, IL-6, α-SMA, TGF-β1, Traf6, and TAK1 in renal tissue of the HT+VD-L, HT+VD-H, and HT+VD-H+pc groups decreased significantly, while the mRNA expression of E-cadherin increased significantly.

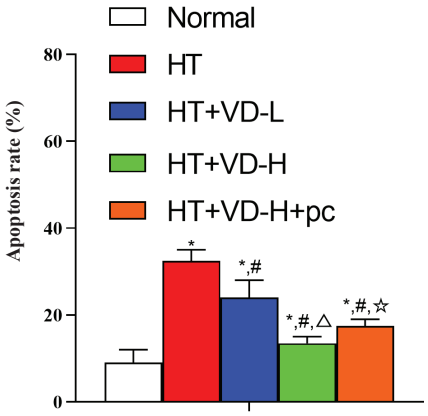

Fig. 3. The cell apoptosis of renal tissue and changes of renal function in rats of each group. Note: One-way ANOVA.Compared with the Normal group, *:p<0.05 compared with the HT group; #:p<0.05 compared with HT+VD-L group; △:p<0.05 compared with HT+VD-H group; ☆:p<0.05).

Compared with the HT+VD-L group, the mRNA expression of TNF-α, IL-6, α-SMA, TGF-β1, Traf6, and TAK1 in the HT+VD-H group decreased significantly, while the mRNA expression of E-cadherin increased significantly. Compared with the HT+VD-H group, the mRNA expressions of TNF-α, IL-6, α-SMA, TGF-β1, Traf6 and TAK1 in the kidney tissue of the HT+VD-H+pc group were significantly increased, while the mRNA expression of E-cadherin was significantly decreased, with statistical significance (all p<0.05), (Fig. 4).

Expression of TNF-α and IL-6 in the renal tissue of rats in each group

The results of immunofluorescence staining showed that compared with the normal group, the fluorescence intensity and expression of TNF-α and IL-6 in the renal tissue of the HT, HT+VD-L, HT+VD-H and HT+VD-H+pc groups increased significantly. Compared with HT+VD-L group, the fluorescence intensity of TNF-α and IL-6 in the HT+VD-H group decreased obviously. Compared with the HT+VD-H group, the fluorescence intensity of TNF-α and IL-6 in the HT+VD-H+pc group increased significantly (Fig. 5).

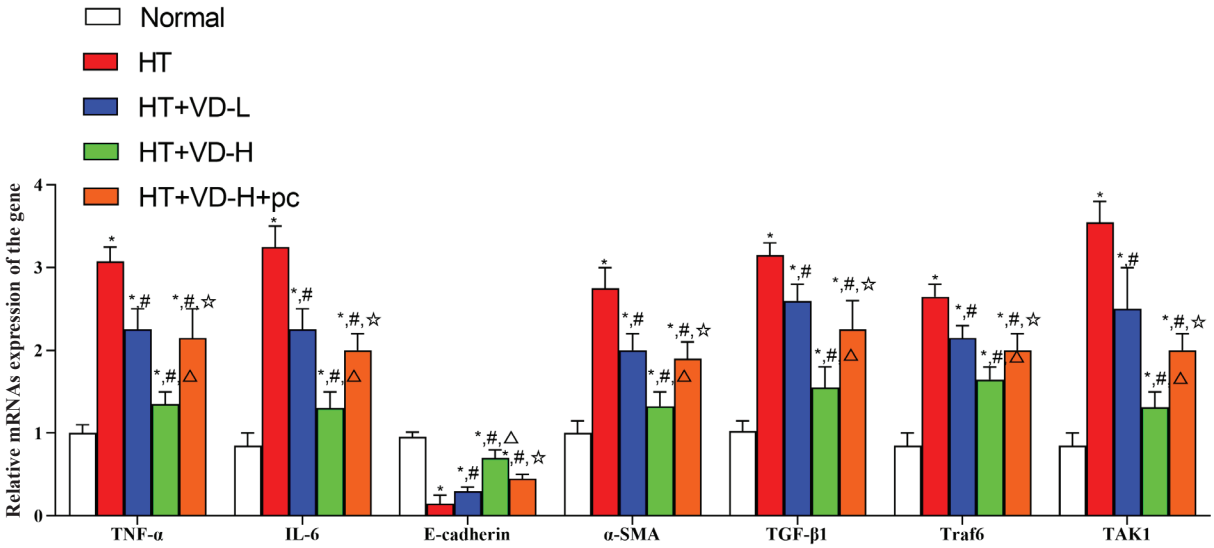

Fig. 4. The expression of related genes in renal tissue in rats of each group (Note: One-way ANOVA. Compared with the Normal group, *:p<0.05 compared with the HT group; #:p<0.05 compared with HT+VD-L group; △:p<0.05 compared with HT+VD-H group; ☆:p<0.05).

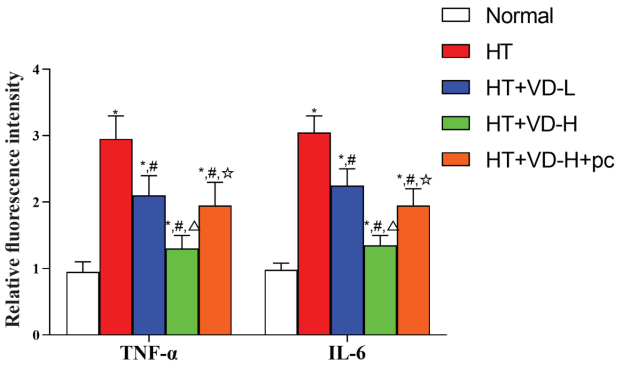

Fig. 5. The expression of TNF-α and IL-6 in renal tissue in rats of each group (Note: One-way ANOVA. Compared with the Normal group, *:p<0.05 compared with the HT group; #:p<0.05 compared with HT+VD-L group; △:p<0.05 compared with HT+VD-H group; ☆:p<0.05).

Expression of E-cadherin, α-SMA and TGF-β1 in the renal tissue of rats in each group

Immunohistochemical results showed that, compared with the normal group, the percentage of α-SMA and TGF-β1 positive cells in the renal tissue of rats in the HT, HT+VD-L, HT+VD-H and HT+VD-H+pc groups increased significantly. In contrast, the percentage of E-cadherin-positive cells decreased significantly, and the expressions of α-SMA, TGF-β1, and E-cadherin increased significantly. Compared with the HT group, the percentage of α-SMA and TGF-β1 positive cells in kidney tissue of the HT+VD-L, HT+VD-H and the HT+VD-H+pc groups decreased significantly, and the percentage of E-cadherin positive cells increased significantly. The expressions of α-SMA and TGF-β1 decreased significantly, and the expression of E-cadherin increased significantly, which was similar to that of HT. In the HT+VD-H group, the percentage of α-SMA and TGF-β1 positive cells in renal tissue decreased significantly, and the percentage of E-cadherin positive cells increased significantly. The expressions of α-SMA and TGF-β1 decreased significantly, and the expression of E-cadherin increased significantly. Compared with the HT+VD-H group, in the HT+VD-H+pc group, the percentage of α-SMA and TGF-β1 positive cells in rat kidney tissue increased significantly. In contrast, the percentage of E-cadherin-positive cells decreased significantly, and the expressions of α-SMA and TGF-β1 increased significantly, with statistical significance (all $p<0.05$)(Fig. 6).

Expression of Bcl-2, Bax, Traf6 and p-TAK1 in renal tissue of rats in each group

Western blot results showed that compared with the normal group, the expressions of Bax, Traf6, and p-TAK1 in renal tissue of rats in HT, HT+VD-L, HT+VD-Hand, and HT+VD-H+pc groups were significantly increased, while the expression of Bcl-2 was significantly decreased. The expressions of Bax, Traf6 and p-TAK1 in the renal tissue of rats in the HT+VD-L, HT+VD-H and HT+VD-H+pc groups decreased significantly, while the expression of Bcl-2 increased significantly. Compared with the HT+VD-L group, the expressions of Bax, Traf6 and p-TAK1 in renal tissue of rats in the HT+VD-H group decreased significantly. In the HT+VD-H+pc group, the expressions of Bax, Traf6 and p-TAK1 in the renal tissue of rats were significantly increased, while the expression of Bcl-2 was significantly decreased, with statistical significance (all $p < 0.05$), while the expression of TAK1 was not statistically significantly different ($p>0.05$), (Fig. 7).

Mechanism of VD alleviating renal injury in HT rats

When HT occurs in rats, Traf6/TAK1 signal in renal tissue is activated, the level of p-TAK1 is increased, the level of transcription and translation of related inflammatory and interstitial fibrosis genes is increased, the level of inflammatory stress in animal renal tissue is increased, and the apoptosis rate of cells is increased, which promote the EMT progress of renal tubular epithelial cells and further affects the renal function

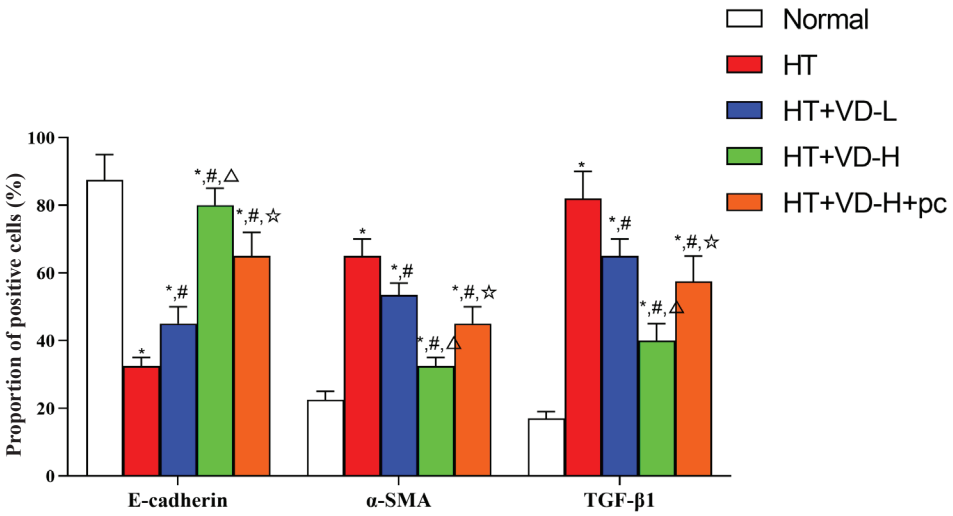

Fig. 6. Percentage of E-cadherin, α-SMA and TGF-β1 positive cells in renal tissue of rats in each group (Note: One-way ANOVA. Compared with the Normal group, *:p<0.05 compared with the HT group; #:p<0.05 compared with HT+VD-L group; △:p<0.05 compared with HT+VD-H group; ☆:p<0.05).

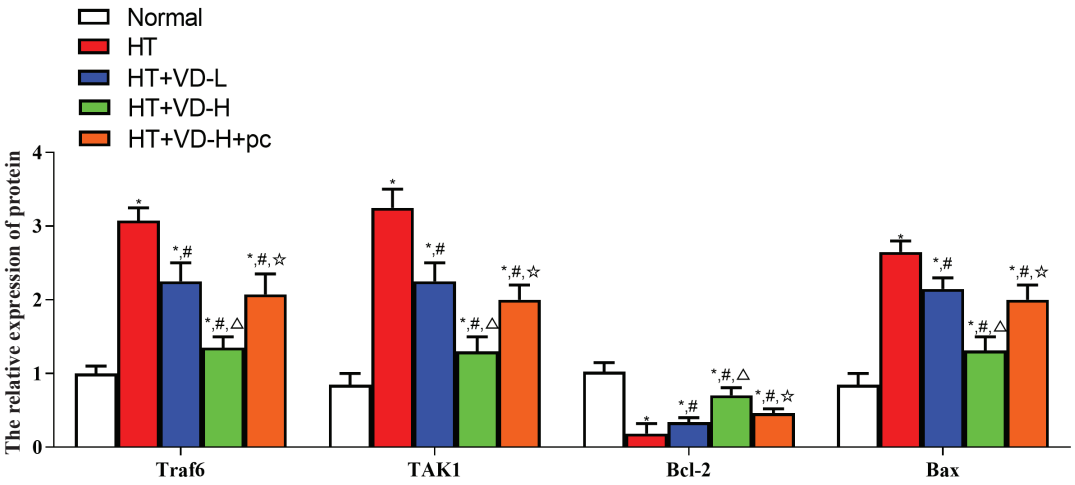

Fig. 7. The expression of Bcl-2, Bax,Traf6, TAK1 and p-TAK1 in renal tissue in rats of each group (Note: One-way ANOVA. Compared with the Normal group, *:p<0.05 compared with the HT group; #:p<0.05 compared with HT+VD-L group; △:p<0.05 compared with HT+VD-H group; ☆:p<0.05).

of animals. Following intervention with VD, the Traf6/TAK1 signal activation was significantly inhibited, leading to decreased levels of p-TAK1, reduced transcription and translation of related genes, lower apoptosis rates in cells within the animal kidney, and limited EMT progression. The rescue experiment was carried out using pCDNA3.1-TAK1, which overexpresses TAK1. The results showed that pcDNA3.1-TAK1 could partially reverse the protective effect of VD on renal tissue, and it was inferred that VD might play a protective role in renal tissue by inhibiting the Traf6/TAK1 signal (Fig. 8).

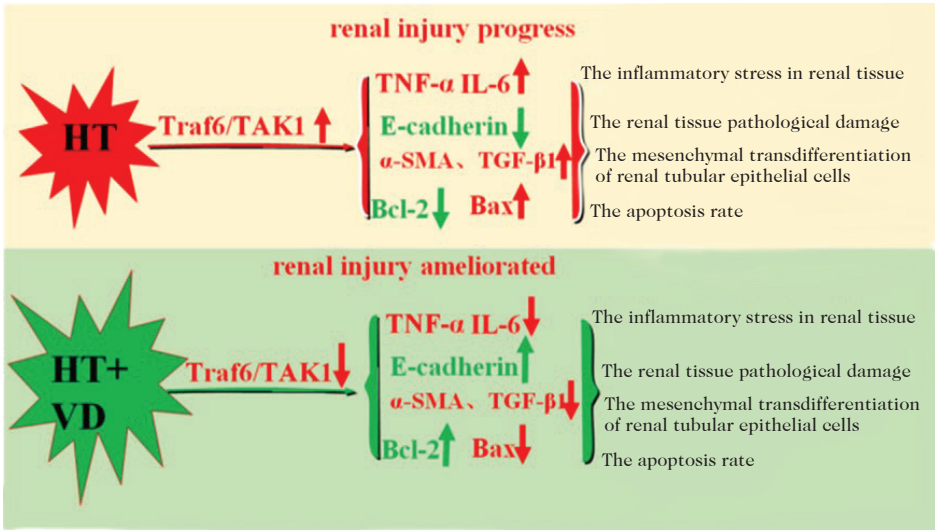

Fig. 8. The schematic diagram of the mechanism of VD attenuating renal injury in HT rats.

DISCUSSION

HT is an endocrine system disorder characterized by a series of metabolic impairments due to insufficient synthesis and secretion of thyroid hormones by the thyroid gland. The pathological mechanisms of this disease are highly complex, affecting multiple organs and tissues such as the liver, kidneys, and brain. Currently, patients could only manage the condition through lifelong oral administration of thyroid hormone supplements, and there is no definitive cure [8]. Renal injury induced by HT is one of the most prominent complications in the progression of the disease. Lathiya et al.[9] indicated that renal injury due to thyroid hormone deficiency was closely related to the progression of renal interstitial fibrosis in patients. Therefore, an in-depth exploration of the molecular mechanisms underlying epithelial-mesenchymal transition (EMT) in renal tubular epithelial cells in HT and the identification of reliable therapeutic targets are of great significance for the clinical treatment of the disease.

Vitamin D (VD), one of the essential vitamins for growth, development, and stress response, primarily participates in bone homeostasis by regulating calcium and phosphorus metabolism in cells. However, recent studies have shown [9] that VD exhibits multiple physiological effects, playing a role in pathophysiological processes such as inflammatory stress, immune regulation, neurotransmitter development, and antitumor activity. A clinical study by Durmuş et al.[10] revealed that supplementing COVID-19 patients with a certain amount of VD significantly reduced their serum levels of α-SMA and TGF-β1, decreased their urine albumin (UAlb) content and serum levels of creatinine (Scr) and blood urea nitrogen (BUN), thereby improving renal dysfunction. Wu et al. demonstrated that exogenous VD supplementation in a model of pituitary secretion deficiency significantly inhibited the expression of TNF-α and IL-6 in renal tissue, downregulated the apoptosis rate in animal renal tissue, and alleviated pathological damage to renal tissue. Li et al. [11] showed that in a model of HK-2 cell injury induced by high glucose, VD intervention significantly inhibited the expression of Bax in cells, increased the expression of E-cadherin, and inhibited the EMT process in cells. In this study, after the construction of an HT model in rats using propylthiouracil (PTU), results from

enzyme-linked immunosorbent assay kits and hematoxylin and eosin (HE) staining of thyroid tissue showed that compared with the normal group, HT rats had significantly elevated serum TSH levels, significantly decreased TT4 levels, and apparent thyroid pathological damage, indicating successful modeling. After VD intervention, renal function parameters (UAlb, Scr, BUN) in HT rats improved significantly, pathological damage to renal tissue was markedly reduced, the apoptosis rate of renal tissue cells decreased significantly, the expression of inflammatory mediators TNF-α and IL-6 in renal tissue was significantly lowered, and EMT in renal tubules was significantly restricted, confirming the protective effect of VD on the kidneys of HT rats.

To better evaluate the interventional effects of drugs and seek reliable therapeutic targets, it was necessary to delve deeper into the drug targets to promote their widespread use in clinical treatment. The activation of Traf6/TAK1 signalling was closely related to the progression of renal diseases such as acute kidney injury, chronic kidney disease, renal aging, and renal cell carcinoma, primarily exerting pro-inflammatory effects, promoting fibrosis, and regulating cell apoptosis and pyroptosis, thereby participating in the occurrence and development of related renal diseases. Wei et al.[12] showed that inhibiting the phosphorylation of TAK1 in a diabetic mouse model significantly reduced the expression of mesenchymal cell marker α-SMA in renal tissue and decreased the percentage of collagen deposition in renal tissue. The results of this study showed that the expression of Traf6 and p-TAK1 was significantly reduced in the renal tissue of rats in both high and low-dose VD groups. Functional rescue experiments using pcDNA3.1-TAK1 overexpressing TAK1 showed that pcDNA3.1-TAK1 could partially reverse the protective effect of VD on renal tissue, suggesting that VD may exert its protective effect on renal tissue by inhibiting Traf6/TAK1 signalling.

In summary, vitamin D can inhibit EMT in renal tubular epithelial cells of HT rats, reduce the apoptosis rate in renal tissue, alleviate pathological damage to renal tissue, and improve renal function. These effects are related to the inhibition of Traf6/TAK1 signalling activation. However, more detailed and systematic research is needed before vitamin D can be comprehensively used in the clinical treatment of the disease.

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Conflicts of interest

The authors declare that they have no conflicts of interest to report regarding the present study.

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Contribution the authors

AC: conceived the study and drafted the manuscript. QH, HW: supervised the project and acquired funding, and critically revised the intellectual content. ZL, YL,JY, HH, RZ, JX, PL: performed the experiments and collected data.

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Clinical effect of laparoscopic surgery on patients with colon cancer complicated with intestinal obstruction.

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Keywords: colon cancer; intestinal obstruction; laparoscopic.

Abstract. Colon cancer is a malignant tumor of the digestive tract, often complicated by intestinal obstruction. Laparoscopic surgery is widely used and has the advantages of a small postoperative wound, less intraoperative blood loss, and fewer postoperative complications. To measure the clinical effect of laparoscopic surgery on patients with colon cancer complicated with intestinal obstruction, the clinical data of 100 patients with this condition, who underwent surgical treatment in the Baoji High-tech Hospital between January 2020 and December 2022, were retrospectively analyzed. Based on different surgical methods, the patients were separated into a control group (CG, traditional laparotomy) and an observation group (OG, laparoscopic surgery). The total clinical effect of OG was superior to that of CG, as evidenced by shorter operation times, reduced intraoperative blood loss, faster recovery times for intestinal function, earlier discharge from bed, and shorter hospital stays. After surgery, the NRS score declined in both groups, with a lower score in the OG. TNF-α, IL-6, and CRP levels were elevated in both groups, but those in OG were lower. The occurrence of complications in the OG was reduced compared to the CG. Quality-of-life scores, including physical function, psychological state, social communication, and self-care ability in the OG, were higher than those in the CG. Laparoscopic surgery is effective for treating colon cancer complicated with intestinal obstruction in patients, which can effectively lessen their pain, reduce their inflammatory indicators, reduce the postoperative complications of patients, and improve their quality of life.

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Efecto clínico de la cirugía laparoscópica en pacientes con cáncer de colon complicado con obstrucción intestinal.

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Palabras clave: cáncer de colon; obstrucción intestinal; laparoscopía.

Resumen. El cáncer de colon es un tumor maligno del tracto digestivo, a menudo complicado por obstrucción intestinal. La cirugía laparoscópica se utiliza ampliamente y presenta ventajas tales como una herida postoperatoria pequeña, menor pérdida de sangre intraoperatoria y menos complicaciones postoperatorias. El objetivo de este estudio fue evaluar el efecto clínico de la cirugía laparoscópica en pacientes con cáncer de colon complicado con obstrucción intestinal. Se analizaron retrospectivamente los datos clínicos de 100 pacientes con cáncer de colon complicado con obstrucción intestinal que se sometieron a tratamiento quirúrgico en el Hospital de Alta Tecnología de Baoji entre enero de 2020 y diciembre de 2022. Según los diferentes métodos quirúrgicos, los pacientes se dividieron en un grupo control (GC, laparotomía tradicional) y un grupo de observación (GO, cirugía laparoscópica). El efecto clínico total del GO fue mejor que el del GC; el tiempo operatorio, la pérdida de sangre intraoperatoria, el tiempo de recuperación de la función intestinal, el tiempo para levantarse de la cama y la estancia hospitalaria fueron menores en el GO. Después de la cirugía, la Puntuación de la Escala de Riesgo Nutricional disminuyó en ambos grupos, siendo más baja en el GO. Los niveles de TNF-α, IL-6 y la Proteína C reactiva se elevaron en ambos grupos, pero fueron más bajos en el GO. La aparición de complicaciones fue menor en el GO que en el GC. Las puntuaciones de calidad de vida, incluyendo función física, estado psicológico, comunicación social y capacidad de autocuidado, fueron más altas en el GO que en el GC. La cirugía laparoscópica es eficaz para tratar a pacientes con cáncer de colon complicado con obstrucción intestinal, ya que puede reducir eficazmente el dolor, los indicadores inflamatorios, las complicaciones postoperatorias y mejorar la calidad de vida de los pacientes.

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INTRODUCTION

Colon cancer is a malignant tumor of the digestive tract that primarily occurs in the colon, particularly at the junction of the sigmoid colon and the rectum [1]. Its incidence is extremely high, ranking as high as the third in the ranking of gastrointestinal tumors [2]. Intestinal obstruction is a relatively common complication of colon cancer[3]. The primary cause of intestinal obstruction in patients with colon cancer is the tumor's narrowing of the intestinal cavity, resulting in dry and hard stool that impedes the passage of intestinal contents [4]. The early symptoms of acute intestinal obstruction are insidious and difficult to detect, and the development of acute intestinal obstruction is rapid after onset, which can easily lead to death [5]. At present, surgery is often used in the clinical therapy of colon cancer complicated with intestinal obstruction [6]. However, traditional laparotomy not only easily leads to large wounds, but is also prone to more complica-

tions, which have adverse effects on the rapid recovery of patients [7,8]. Laparoscopic surgery has been widely used in the surgical therapy of colon cancer complicated with intestinal obstruction patients due to its advantages of small postoperative wound, less intraoperative blood loss, fewer postoperative complications, and quick postoperative recovery [9]. The objective of this study was to investigate further the effect of laparoscopic surgery on patients with colon cancer complicated with intestinal obstruction.

MATERIALS AND METHODS

Patients

The clinical data of 100 colon cancer patients complicated with intestinal obstruction who underwent surgical treatment in our hospital from January 2020 to December 2022 were retrospectively analyzed. Based on different surgical methods, the patients were separated into a control group (CG) and an observation group (OG), with 50 cases in each group.

Inclusion criteria: (1) Patients diagnosed with colon cancer combined with intestinal obstruction; (2) The patient had not received any other treatment before surgery.

Exclusion criteria: (1) Patients with malignant tumors of other sites; (2) Patients with abnormal heart, liver and kidney function; (3) Patients who had received open surgery; (4) Intestinal perforation. No significant difference was discovered in baseline data between the two groups ($p>0.05$), reflecting comparability, as shown in Table 1.

Treatments

Both groups underwent general anesthesia before surgery, and artificial pneumoperitoneum with a pressure of about 15 mmHg was established. Different surgical positions were taken according to the different locations of the colon cancer tumor and intestinal obstruction.

The CG received a traditional laparotomy. The surgical approach was to make an incision in the middle of the lower abdomen of the patient. The incision was made successively according to subcutaneous tissue levels, and the abdominal tumor and intestinal obstruction sites were carefully observed to determine their size, location, and adjacent tissues, to select the resection method for resection of the tumor, intestine and lymph nodes. After resection, the bleeding status of the patient was checked, and abdominal cleaning was performed. The surgical incision was sutured layer by layer, and the drainage tube was placed. After laparotomy, the patients were treated with routine anti-infection therapy and fluid rehydration.

The OG was treated with laparoscopic surgery. Puncture was performed on the left and right sides below the belly button of the patient, with a length of about 10 mm. The laparoscope was placed in the abdomen of the patient, and the abdominal tumor and intestinal obstruction were observed under the laparoscope. Then, the left and right sides of the upper abdomen of the patient were selected for puncture, and a Trocar with a length of about 5 mm was placed in them. The size, location, and adjacent tissues of the tumor

Table 1. General data of patients in both groups.

Indicators		Control group (n=50)	Observation group (n=50)	p
Gender (male/female)		30/20	29/21	>0.05
Average age (years)		52.93±8.35*	53.06±8.47*	>0.05
TNM stage	Stage I	20	21	>0.05
	Stage II	25	24	
	Stage III	5	5	

* Mean ± standard deviation. TNM: tumor/node/metastasis.

and intestinal obstruction were determined under the laparoscope, and the primary and secondary operation holes were established according to them. Thus, intestinal adhesion lysis was performed, the tumor intestines and lymph nodes were removed, and the abdominal cavity of the patient was rinsed with normal saline, and the surgical incision was sutured layer by layer. After laparoscopic surgery, the patients were treated with routine anti-infection and fluid rehydration.

Observation indicators

(1) Evaluation of clinical effects. *Cure*: after treatment, the patient's clinical symptoms and pathological tumor disappeared, X-ray examination showed no intestinal dilation in the abdomen, incision healing without complications; *Improvement*: the clinical symptoms were significantly improved, the lesion and tumor were reduced by more than half, and the abdominal intestinal obstruction was partially relieved by X-ray examination. *Ineffective*: those who do not meet the above criteria or whose disease worsens. Total effective rate = cure rate + improvement rate.

(2) Evaluation of surgical indicators. The operation time, intraoperative blood loss, recovery time of intestinal function, time of getting out of bed and hospital stay of patients were observed and recorded.

(3) Pain score was evaluated using a numerical rating scale (NRS). The total score was 0-10 points.

(4) Inflammatory factors. 5 mL of fasting peripheral blood was gathered from patients before and three days after surgery in the morning, respectively. Serum was collected after centrifugation, and the serum levels of TNF-α, IL-6, as well as CRP, were examined employing double-antibody sandwich enzyme-linked immunosorbent assay (ELISA).

(5) The occurrence of complications, including pulmonary infection, incision infection, intra-abdominal hemorrhage and anastomotic fistula in both groups was compared.

(6) The postoperative quality of life score of the two groups was compared, including: physical function, psychological state, social communication, as well as self-care ability, 25 points for each item, a total score of 0 ~ 100 points.

Statistical analysis

This experiment was conducted with SPSS 22.0 statistical analysis software. The measurement data of normal distribution were exhibited as (x±sd), and the t-test was adopted for analysis. The count data were expressed as a rate (%), and a χ^2 test was performed between groups, $p<0.05$ meant the difference was statistically significant.

RESULTS

Clinical effect

Table 2 displayed that the total clinical effect of the OG presented better when comparing with the CG ($p<0.05$).

Surgical indicators in both groups

The operation time, intraoperative blood loss, recovery time of intestinal function, time of getting out of bed and hospital stay of patients in the OG presented shorter relative to the CG (Table 3).

Table 2. Clinical effect.

Groups	N	Cure	Improvement	Ineffective	Total effective rate
Control group	50	20	22	8	42 (84.00%)*
Observation group	50	26	23	1	49 (98.00%)
$\chi2$					5.983
p					<0.05

* Data expressed as n(%).

Table 3. Surgical indicators.

Indicator	Control group	Observation group	p*
Operation time (min)	120.0 ± 13.4*	90.0 ± 11.6	<0.05
Intraoperative blood loss (mL)	300.0 ± 45.3	150.0 ± 30.2	<0.05
Recovery of intestinal function (days)	4.2 ± 0.7	2.8 ± 0.6	<0.05
Time of getting out of bed (days)	2.3 ± 0.5	1.5 ± 0.4	<0.05
Hospital stay (days)	9.6 ± 1.4	6.2 ± 1.0	<0.05

Data expressed as mean±standard deviation *t-Student test.

Degree of pain

No difference was seen in NRS score between the two groups before surgery (p>0.05). After surgery, the NRS score declined in both groups, and that in the OG was lower when compared with the CG (Table 4).

Table 4. Degree of pain.

Group	NRS (Pre-op)	NRS (Post-op)	p*
Control group	6.5 ± 0.9	4.2 ± 0.7	<0.05
Observation group	6.4 ± 1.0	2.7 ± 0.6	<0.05

NRS: numerical rating scale. Data expressed as mean ± standard deviation * t-Student test.

Inflammatory response

No difference was seen in TNF-α, IL-6, and CRP levels between the two groups before surgery (p>0.05). After surgery, TNF-α, IL-6, and CRP levels were increased in both groups, but those in the OG presented lower when compared with the CG (Table 5).

Occurrence of complications

Table 6 displayed that the occurrence of complications in the OG was lower when compared with the CG (p<0.05).

Quality of life

After surgery, the quality of life scores, including physical function, psychological state, social communication, as well as self-care ability in the OG, were higher when compared with the CG (Table 7).

Table 5. Inflammatory response.

Marker	CG Pre-op	CG Post-op	OG Pre-op	OG Post-op	p*
TNF-α (pg/mL)	15.3 ± 2.1	35.6 ± 4.5	15.1 ± 2.0	28.4 ± 3.6	<0.05
IL-6 (pg/mL)	24.1 ± 3.2	49.8 ± 5.3	23.9 ± 3.4	36.2 ± 4.0	<0.05
CRP (mg/L)	18.7 ± 2.8	44.2 ± 5.8	18.4 ± 2.9	30.1 ± 4.7	<0.05

CG: Control group, OG: Observation group. Data expressed as mean±standard deviation* t- Student test between Pre-op and Post-op.

Table 6. Occurrence of complications.

Groups	N	Pulmonary infection	Incision infection	Intra-abdominal hemorrhage	Anastomotic fistula	Total incidence rate
Observation group	50	1	0	1	1	3 (6.00%)*
Control group	50	3	2	3	3	11 (22.00%)
χ^2						5.316
p						<0.05

*Data expressed as n(%).

Table 7. Quality of life

Domain	Control group	Observation group	p*
Physical function	17.5 ± 2.0	22.4 ± 1.8	<0.05
Psychological state	18.2 ± 1.9	23.1 ± 2.1	<0.05
Social communication	17.9 ± 2.3	22.6 ± 1.7	<0.05
Self-care ability	18.0 ± 2.4	23.2 ± 1.9	<0.05

Data expressed as mean±standard deviation *t-Student test.

DISCUSSION

Intestinal obstruction is one of the most common clinical complications of colon cancer, the cause of which is closely related to postoperative infection and intestinal adhesion in patients with this condition [9]. The clinical symptoms are often manifested as abdominal distension, constipation and vomiting, etc. [10] Because the early symptoms of intestinal obstruction are not easy to detect, and the development rate after the onset of the disease is fast, it has a significant adverse influence on the survival, quality of life and postoperative recovery of patients [11].

At present, surgery is usually used in the clinical therapy of colon cancer complicated with intestinal obstruction, and the curative effect is exact; the tumor can be removed in one time, and the obstruction can be removed in one time [12]. Traditional laparotomy is the leading choice for the clinical therapy of colon cancer complicated with intestinal obstruction, which has good therapeutic effect and can effectively remove the tumor and relieve the intestinal obstruction of patients [13]. However, the traditional open surgery will leave a large wound and multiple postoperative complications, resulting in a slow postoperative recovery [14]. Therefore, in the therapy of colon cancer patients with intestinal obstruction, it is imperative to adopt a surgical treatment with minor postoperative wounds, fewer postoperative complications, and rapid postoperative recovery, which not only improves the survival rate of patients but also promotes their quality of life.

In recent years, minimally invasive surgery has been extensively applied in abdominal surgery, and laparoscopic surgery, as a minimally invasive surgery, has been widely used in clinical treatment for its advantages of small postoperative wound, less intraoperative blood loss, fewer postoperative complications and quick postoperative recovery [15]. In treating colon cancer complicated with intestinal obstruction in patients, laparoscopic surgery can be used to observe the patient's abdominal cavity through a video probe [16]. At the same time, the magnification of laparoscopy can effectively ensure the surgical field of view, so that the patient's lesion area is fully and clearly exposed [17]. Moreover, laparoscopy offers the advantage of multi-angle exploration, allowing for the clear exposure of positions that are not readily observable in traditional open surgery, thereby facilitating detailed and precise surgical operations [18]. In addition, laparoscopic surgery can effectively decrease operation time, reduce postoperative wounds, and decrease intraoperative blood loss and postoperative complications, thereby speeding up the patient's recovery [19].

Our study indicated that the total clinical effect of the OG was better when compared with the CG. The operation time, intraoperative blood loss, recovery time of intestinal function, time of getting out of bed and hospital stay of patients in the OG were shorter relative to the CG. The NRS score declined in both groups, with the OG presenting lower scores when compared to the CG. All these outcomes indicated that the application of laparoscopic surgery could shorten the length of hospital stay, reduce the amount of intraoperative blood loss, alleviate the pain of patients, improve the efficiency of clinical treatment, and promote the rehabilitation process of patients in treating colon cancer complicated with intestinal obstruction. Consistently, Ruben Veldkamp et al. [20] have indicated that lapa-

roscopic colectomy is linked to earlier recovery of bowel function, a lower requirement for analgesics, and a shorter hospital stay relative to open colectomy [20].

During surgical trauma, patients would also activate the inflammatory response and promote the secretion of inflammatory factors [21]. TNF-α is a pro-inflammatory factor, which is secreted by mononuclear macrophages. IL-6 is a crucial cytokine that regulates intercellular immunity and cooperates with other cytokines in patients to transmit an inflammatory response, serving as a key indicator for evaluating the degree of surgical trauma in patients [22]. CRP is an important mediator of acute inflammation. When patients suffer from surgical trauma, the level of their pain will be significantly increased, which dramatically improves the tissue repair ability of patients [23]. However, research in this field has found that CRP levels in patients are positively linked to the degree of surgical trauma [24]. Our study indicated that after surgery, TNF-α, IL-6, and CRP levels increased in both groups. However, those in the OG were lower when compared with the CG, suggesting that the use of laparoscopic surgery could inhibit the inflammatory response in colon cancer patients complicated with intestinal obstruction. Consistently, it has been reported that the inflammatory response is lower in laparoscopic rectal surgery when compared with conventional open surgery [25].

In addition, our study indicated that the occurrence of complications in the OG was lower when compared with the CG, and the quality of life scores, including physical function, psychological state, social communication, as well as self-care ability in the OG, were higher when compared with the CG. All above outcomes indicated that the application of laparoscopic surgery could reduce the complications and promote the quality of life of patients with colon cancer complicated with intestinal obstruction, which was in agreement with previous studies [26]. As a conclusion, laparoscopic surgery significantly impacts the treatment of colon cancer patients with intestinal obstruction, effectively reducing patient pain, lowering inflammatory indicators, minimizing postoperative complications, and improving the patient's quality of life.

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Ethical considerations

The Medical Ethics Committee of the Baoji High-tech Hospital approved this work.

Conflict of interests

There are no conflicts of interest in this study.

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Author's contributions

PW was responsible for concepting the study, collecting, analyzing data, and drafting the manuscript. ZT revised the manuscript and managed the data.

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Condiciones comórbidas del síndrome de West y autismo: Reporte de 5 casos.

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Palabras clave: síndrome de West; espasmos infantiles; trastornos del espectro autista (TEA).

Resumen. El síndrome de West (SW) es una encefalopatía epiléptica caracterizada por espasmos infantiles (EI), retraso o regresión psicomotora, y un patrón típico electroencefalográfico denominado hipsarritmia. Los EI son crisis tónicas de aparición súbita, en flexión, extensión o mixtas, de breve duración y en serie, que se presentan habitualmente en el primer año de la vida. Se estima que al menos 20 por ciento de los niños con SW desarrollan autismo. Tanto el SW como el trastorno del espectro autista (TEA) comparten alteraciones genéticas, estructurales y metabólicas, así como disfunción inmunológica, que interactúan con factores ambientales, lo que sugiere posibles mecanismos comunes que vinculan ambos trastornos. Se realizó un análisis descriptivo, retrospectivo, de los registros clínicos de cinco niños de edades comprendidas entre 3 y 7 años, con EI y signos clínicos del TEA. A todos se les realizó evaluación neurológica y psicológica, y electroencefalograma (EEG). La edad promedio fue de 4,4 años con predominio del sexo masculino. El inicio de los EI ocurrió entre los 4 y 7 meses de edad. Los signos autistas se identificaron entre 1 y 2 años y medio y en todos los pacientes el EEG mostró hipsarritmia. Tres de los cinco niños tenían diagnóstico de síndrome neurocutáneo, y todos fueron tratados con ACTH y/o nitrazepam, con una respuesta satisfactoria. Se destaca la relación con los síndromes neurocutáneos, particularmente la esclerosis tuberosa, donde probables mecanismos celulares, moleculares y fisiopatológicos, afectan la conectividad neuronal, determinando el proceso epileptogénico y la demora del desarrollo psicomotor. La identificación temprana de estos signos clínicos por parte del pediatra, permitirá realizar intervenciones oportunas.

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Comorbid conditions of West syndrome and autism: Report of five clinical cases.

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Keywords: West syndrome; infantile spasms; autism spectrum disorders (ASD).

Abstract. West syndrome (WS) is an epileptic encephalopathy characterized by infantile spasms (IS), psychomotor delay or regression, and a typical EEG pattern called Hypsarrhythmia. IS are tonic seizures of sudden onset, in flexion, extension, or mixed, of short duration, and in series, which usually occur in the first year of life. It is estimated that at least 20 percent of children with WS develop autism. Both WS and autism spectrum disorders (ASD) share genetic, structural, and metabolic alterations, as well as immune dysfunction, which interact with environmental factors, suggesting potential common mechanisms linking both disorders. We performed a retrospective and descriptive analysis of clinical records of five children between the ages of three and seven years, all presenting with IS and clinical signs of ASD. Comprehensive neurological and psychological assessments were conducted, along with EEGs. The average age was 4.4 years, with a predominance of males. The onset of symptoms occurred between four and seven months, and signs of autism were identified between one and 2.5 years. All patients exhibited hypsarrhythmia on their EEGs. Three of the five children had a diagnosis of neurocutaneous syndrome. All were treated with ACTHH or nitrazepam, with a satisfactory response. We highlight the relationship with neurocutaneous syndromes, particularly tuberous sclerosis, where cellular, molecular, and pathophysiological mechanisms may impact neuronal connectivity, thus influencing the epileptogenic process and developmental delay. Early identification of these clinical signs by the pediatrician will allow for timely interventions.

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INTRODUCCIÓN

El síndrome de West (SW) se define como una encefalopatía epiléptica dependiente de la edad, caracterizada por una tríada electroclínica, compuesta por espasmos infantiles (EI), retraso o regresión en el desarrollo psicomotor, y un trazado caótico en el electroencefalograma (EEG). Los EI se presentan habitualmente en el primer año de la vida como contracciones tónicas de pocos segundos de duración, que interesan principalmente la musculatura del cuello, tronco y miembros, con abducción o aducción de los brazos. Los episodios son bilaterales y simétricos, aparecen en serie, en secuencia rápida, justo antes de dormir o al despertar, y pueden ir acompañados de llanto o grito, antes o después de los espasmos; durante la crisis puede haber desviación o fijación de la mirada [1,2]. El patrón anormal del EEG se denomina hipsarritmia, y se caracteriza por descargas paroxísticas interictales, con ondas lentas de alto voltaje y puntas de localización variable, que ocurren en forma continua o en salvas de distinta duración [3-7].

Los EI ocurren en cerca de 0,249 casos por cada 1000 nacidos vivos, con una prevalencia general de 1/10.000 niños, a la edad de 10 años[1]. Se ha señalado una incidencia de 1,6 por cada 100.000 personas y, aproximadamente el 20% de los pacientes con SW presentan TEA asociado [1,8]. Los nuevos enfoques en la metodología de estudio de los pacientes con EI originaron una nueva nomenclatura. Se incorporó el término espasmos epilépticos, tomando en consideración la variedad de fenotipos complejos y de registros del EEG, así como también las anomalías genéticas, la patogenia, la demora del desarrollo, las opciones de tratamiento y el pronóstico [1]. Los espasmos epilépticos pueden presentarse como fenotipos atípicos electroclínicos, ya sea bajo la forma de espasmos sutiles, hipsarritmia modificada o con un comienzo fuera de la infancia [6,9]. Estos espasmos forman parte del denominado "síndrome de espasmos infantiles (SEI)" que se asocia con eventos estructurales, infecciosos, metabólicos e inmunológicos, que interactúan bajo una base genética, y se presentan como asociaciones complejas [1,9-12]. El SW representa el 90% de los casos de SEI [1,8]. En la actualidad, los términos clásicos SW y EI continúan siendo los más citados en la literatura.

El autismo, mejor identificado como el trastorno del espectro autista (TEA), es una discapacidad del desarrollo que afecta la manera en la que una persona percibe y socializa con otras personas, lo que causa problemas en la interacción social y la comunicación[13,14]. Esta condición también comprende patrones de conducta restringidos y repetitivos con criterios definidos propuestos en el Manual Diagnóstico y Estadístico de los Trastornos Mentales (DSM5) [8]. Estimaciones recientes señalan que uno de cada 36 niños de 8 años tiene el espectro autista [15]. Esta afección también impacta diversas áreas, como la velocidad de procesamiento, aprendizaje y memoria verbal, razonamiento y resolución de problemas [8].

En comparación con la población general, diversos trastornos epilépticos se presentan con mayor frecuencia en personas con TEA. Se señala una alta prevalencia del autismo en ciertas encefalopatías epilépticas como el SW, una conexión de gran interés porque el estudio conjunto de estos dos trastornos puede mejorar nuestra comprensión de los mecanismos genéticos, moleculares y celulares implicados, así como conducir a mejores terapias para ambas afecciones.

La prevalencia de autismo en los niños que presentan epilepsia es cercana al 35%[16]. Juntos, TEA y Epilepsia tienen una prevalencia de 0,6% en la población general [15]. La comorbilidad de estos dos cuadros clínicos es de grave perfil y se relaciona con déficits que abarcan cualquier área del neurodesarrollo [10,11]. Ambos trastornos comparten alteraciones de ciertos mecanismos cerebrales, tales como la transcripción genética, el crecimiento celular, la sinaptogénesis y los sistemas glutamatérgicos y gabaérgicos[1,8,10,15,16]. El gen SCN2A codifica una proteína relacionada con los canales de sodio, que es esencial para transmitir señales eléctricas en el sistema nervioso. Las variaciones en este gen se han asociado con una variedad de trastornos del desarrollo neurológico y encefalopatías epilépticas, incluyendo el SW [17-22].

Existe gran dificultad para reconocer el verdadero comienzo de los EI, porque estos, a menudo, suelen ser sutiles. Igualmente, es difícil evaluar el desarrollo psicomotor de un niño al inicio de los espasmos. Algunos autores no mencionan la evidencia previa de demora del desarrollo para definir el SW, como fue inicialmente descrito, pues muchos niños comienzan los espasmos tras un desarrollo psicomotor normal [1]. En este trabajo se presentan cinco pacientes venezolanos con la asociación de SW con TEA, se describen tanto las manifestaciones clínicas como su evolución y se discuten algunos mecanismos genéticos, moleculares, celulares y terapéuticos. El diagnóstico temprano de estos trastornos, el cual es principalmente clínico, obtenido mediante la cuidadosa exploración física y una búsqueda minuciosa de sus di-

versas manifestaciones garantizará un tratamiento oportuno y un mejor pronóstico.

Reporte de casos

Se realizó un estudio descriptivo, retrospectivo, de los registros de cinco niños, atendidos en la Consulta de Pediatría, del Centro Médico de Occidente y en la Consulta de Neuropediatría del Hospital Clínico de Maracaibo, Venezuela. Se evaluaron las siguientes características de los pacientes: edad, sexo, antecedentes perinatales, comienzo de las manifestaciones clínicas, trastornos neurológicos asociados, hallazgos en el EEG, tratamiento recibido y evolución clínica. A todos se les realizó una evaluación neurológica, psicológica y electroencefalográfica (Tabla 1). Cuatro pacientes eran de sexo masculino y uno de sexo femenino, con edades comprendidas entre 3 y 7 años (edad promedio: 4,4 años). Todos los pacientes presentaron EI en los primeros meses de vida; en cuatro de ellos, estos episodios ocurrieron antes de los 6 meses. Los signos clínicos de TEA se identificaron entre el primer y segundo año de vida. Dos pacientes presentaban lesiones acrómicas en la piel y uno de ellos exhibía manchas hipercrómicas; un paciente tenía historia de hipoxia feto-neonatal. En los cinco niños se evidenciaron signos de deterioro neuropsíquico.

Se observó una variabilidad en cuanto a la etiología subyacente; tres de los cinco niños recibieron un diagnóstico clínico de síndrome neurocutáneo (dos pacientes con esclerosis tuberosa y uno con neurofibromatosis). Los dos pacientes restantes fueron diagnosticados con un error innato del metabolismo e hipoxia perinatal, respectivamente.

Todos los pacientes presentaron un trazado hipsarrítmico en el EEG. En cuanto al tratamiento indicado, nuestros niños recibieron ACTH y/o nitrazepam. Tres de ellos se trataron con ACTH (como fármaco inicial disponible) y nitrazepam. Dos pacientes

Tabla 1. Características clínicas, electroencefalográficas, tratamiento recibido y evolución de los pacientes.

Características	Casos clínicos				
	1	2	3	4	5
Sexo	Femenino	Masculino	Masculino	Masculino	Masculino
Edad (años)	3	5	4	3	7
Edad de inicio de los espasmos (meses)	5	7	5	5	4
Edad de inicio de los signos autistas (meses)	30	24	12	24	12
Etiología	Acidosis láctica	Esclerosis Tuberosa	Hipoxia perinatal	Esclerosis Tuberosa	Neurofibromatosis
Deterioro neuropsíquico	+	+	+	+	+
Hipsarritmia	+	+	+	+	+
Tratamiento recibido	ACTH + Nitrazepam	ACTH + Nitrazepam	Nitrazepam	ACTH + Nitrazepam	Nitrazepam
Cese de espasmos	+	+	+	–	+
Recurrencia	–	–	–	+	–

ACTH: hormona adrenocorticotrópica.

recibieron nitrazepam como monoterapia, con un resultado favorable desde el punto de vista de remisión de los EI. Los pacientes respondieron adecuadamente a la medicación, con desaparición de los espasmos en las primeras dos semanas de tratamiento. En uno de nuestros pacientes, con diagnóstico de esclerosis tuberosa, se presentó recurrencia de los EI, por lo que necesito reiniciar el tratamiento con ACTH. Al analizar los EEG de todos estos pacientes, se identificó inicialmente el trazado hipsarrítmico, el cual se modificó después del primer año en todos los casos (Tabla 1).

DISCUSIÓN

Nuestro estudio confirma la asociación del SW y el TEA. Destaca también su relación con los síndromes neurocutáneos, particularmente la esclerosis tuberosa, cuya causa genética está bien reconocida. Con base en estos hechos, hay suficientes indicios de que los espasmos infantiles y los fenotipos relacionados pueden ser el resultado de alteraciones en las vías genéticas, además de eventos adquiridos, incluida la hipoxia perinatal [8,9,15]. Estos factores son determinantes para afectar el desarrollo cerebral y ocasionar las diversas manifestaciones clínicas observadas en ambos trastornos. La alteración estructural y funcional de las sinapsis, es un rasgo característico en ciertas formas de epilepsia como el SW y también del TEA [1].

En relación con la edad, los hallazgos obtenidos en este estudio son similares a los reportados en la literatura, los cuales señalan también un predominio en los varones [1-4]. Algunos estudios informan que la prevalencia del sexo masculino es relativamente pequeña [1]. Otros, en cambio, refieren un predominio en las niñas [10]. No obstante, en la mayoría de los estudios demográficos, el autismo es alrededor de cuatro veces más frecuente en los varones [2,8,13-18].

La causa más comúnmente identificada en esta investigación, con una pequeña serie de pacientes, fue el "Síndrome neurocutáneo", lo que contrasta con lo reportado por otros autores quienes señalan como causa más frecuente la "Encefalopatía hipóxico-isquémica" [1,19]. Se hace necesario estudiar un mayor número de pacientes para corroborar estos hallazgos. La observación de tres niños con síndrome neurocutáneo enfatiza la necesidad de realizar un examen clínico detallado y cuidadoso de la piel y anexos en los lactantes con SW, y en aquéllos que exhiban signos que traduzcan una demora del desarrollo psicomotor, o signos sugestivos del TEA (Tabla 2). La piel de estos niños puede revelar manchas, parches y otros cambios que son característicos de estos trastornos (Fig. 1). La Esclerosis tuberosa, una afección médica en la que la vía mTOR está hiperactiva, se caracteriza por un desequilibrio entre las señales excitadoras e inhibidoras, causado por una síntesis anormal de proteínas. Las mutaciones de los genes TSC1 y TSC2, son la causa principal de este trastorno, que provoca disrupción neuronal e interneuronal originando malformaciones, como la formación de tubérculos corticales [20-22]. Estos cambios estructurales pueden provocar trastornos del neurodesarrollo como TEA y convulsiones difíciles de controlar, lo que anuncia un pronóstico grave. Las personas con esta afección tienen un 50% de posibilidades de desarrollar TEA y más del 80% de probabilidad de experimentar epilepsia [1].

Aunque el TEA y el síndrome de West son trastornos neurológicos distintos, con patologías diferentes, pueden compartir mecanismos fisiopatológicos similares, en particular durante la infancia. Los fenómenos neurobiológicos subyacentes, presentes en ambas afecciones, están asociados[23,24,25]. Los procesos epigenéticos moleculares como la metilación del DNA, la modificación de las histonas, la remodelación cromosómica y la regulación mediada por el RNA no codificante causan variantes genéticas que guardan estrecha conexión y son además influenciados por factores ambientales [26,27].

Tabla 2. Signos tempranos de autismo.

Conducta	Edad		
	12 meses	18 meses	24 meses
Social/ emocional	-Escaso contacto ocular -Escasez de sonrisa -No orientación al nombre -No sigue la mirada de otros -Iniciativa social pobre -Escasa expresión facial -Poca regulación emocional -Escasez posturas anticipatorias	-Aversión a la mirada -Escasa expresión emocional -No respuesta al nombre -Menos cambios de atención entre objetos y personas -Poca atención a gestos y/o cambios de atención de otros -Falta de imitación -Poca atención al malestar de otros	Falta de interés social y en otros niños -Contacto ocular muy breve -Poca variedad de expresiones afectivas -No ofrece consuelo
Comunicativa/ simbolica	-Poca frecuencia de vocalizaciones -Escasa respuesta a la atención conjunta -Escasez de actos de señalar -Ausencia de actos de mostrar -Retraso de balbuceo	-No señala para pedir -Pocas respuestas e inicios de atención conjunta -Pocas consonantes comunicativas -Pocos gestos y/o poco variados -Retraso en lenguaje receptivo y expresivo -Poco juego y poco variado	-Pocas respuestas a la atención conjunta -Poca integración de mirada y comunicación -No busca a otros para compartir intereses -Pocos gestos -Escaso vocabulario -Prosodia atípica
Atencional/ sensoriomotora	-Déficit en el desenganche atencional -Movimientos poco variados/atípicos -Hipotonía -Anormalidades de activación y en respuestas sensoriales -Escasa coordinación -Pasividad y escasa conducta exploratoria -Patrón atencional anormal	-Conductas estereotípadas	-Conductas repetitivas e intereses restrictivos

Tomado de Torres-López DA. Detección temprana del trastorno del espectro autista en la escuela. 2019. Eduscientia. Divulgación de la ciencia educativa. 17-23 (Referencia #38).

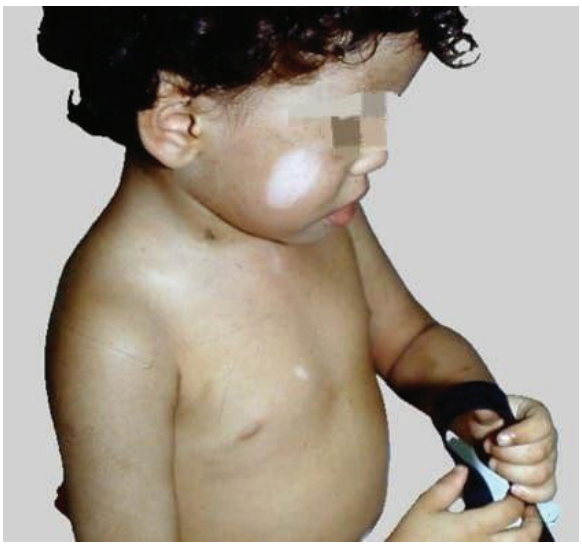

Fig. 1. Típicas manchas acrómicas en un paciente con esclerosis tuberosa.

Diversos estudios señalan que ciertas mutaciones genéticas generan anomalías en la sinapsis y una inestabilidad entre la inhibición y la excitación neuronal (conectividad). Esto se debe a la alteración de las proteínas involucradas en todas las fases de la excitabilidad, mecanismos íntimamente relacionados con el desarrollo del TEA y la epilepsia [28-30]. Hay evidencia de que los genes implicados alteran la sinapsis de forma estructural y funcional confiriendo un alto riesgo de desencadenar convulsiones de comienzo temprano como los EI y TEA[31-35].

En cuanto al tratamiento, aunque la ACTH sigue siendo la medicación de elección para el SW en diversos centros de estudio [36-38], en la actualidad, existen nuevas alternativas terapéuticas, algunas con rela-

tiva especificidad etiológica. El desarrollo de estrategias basadas en terapia molecular busca prevenir los efectos adversos de estos trastornos graves. Los fármacos antagonistas de la via mTOR, como Rapamicina y Everolimus, se han propuesto como una novedosa opción terapéutica para pacientes con esclerosis tuberosa, cuyas anomalías estructurales cerebrales condicionan resistencia a drogas anticonvulsivantes, trastornos del neurodesarrollo y graves defectos cognitivos. La terapia con everolimus, disminuye radicalmente la frecuencia de las convulsiones, en pacientes con esclerosis tuberosa, con un aumento aparente en las habilidades del desarrollo. En el caso de los anticonvulsivantes, un alto porcentaje de pacientes con esclerosis tuberosa es altamente resistente a la medicación, lo que hace necesario el uso de terapias no farmacológicas. El Vigabatrin, un medicamento antiepiléptico inhibidor de la GABAtransaminasa, se considera la primera opción para los EI asociados con este trastorno [17,39,40]. No obstante, los mecanismos que conducen a la epilepsia en la esclerosis tuberosa son complejos y están influenciados por diversos factores, lo que puede provocar una respuesta deficiente al tratamiento. Por ello, la terapia hormonal y la vigabatrina se reconocen actualmente como terapias de primera línea o estándar para esta afección. El seguimiento a largo plazo de estos pacientes puede identificar nuevas recurrencias de espasmos, que podrían requerir el uso de otras alternativas terapéuticas. Esto podría sospecharse en uno de nuestros pacientes con esclerosis tuberosa, como mencionamos previamente.

El pronóstico del SW suele ser reservado, la etiología genética o anatomo-estructural predispone a deterioro intelectual. Los siguientes factores representan un alto riesgo para los niños: edad menor a los seis meses al comienzo de los EI, retardo del desarrollo psicomotor previo a los espasmos, comienzo tardío del tratamiento, pobre respuesta terapéutica y sus efectos adversos, los cuales ensombrecen el futuro de estos pacientes [24,40,41].

Desde el primer reporte hecho por West en 1841 sobre los EI en su propio hijo, la evolución de los avances diagnósticos y terapéuticos se ha incrementado considerablemente [42]. Existen múltiples causas genéticas y ambientales tanto para el TEA como para el SW, y ambos pueden conceptualizarse como trastornos de conectividad aberrante. Las convulsiones en etapas tempranas de la vida, pueden involucrar una alteración de la función de los sistemas de neurotransmisores y de las propiedades neuronales intrínsecas durante el neurodesarrollo, lo que conduce directamente a una interrupción de la conectividad cortical. Las manifestaciones clínicas de esta alteración son las convulsiones o cambios devastadores de la comunicación social y el comportamiento, o ambos. Se necesitan más investigaciones en modelos animales con epilepsia y TEA para resolver muchas preguntas sin respuesta sobre esta importante asociación. El desarrollo de nuevas terapias representa una esperanza para esta condición grave. Es esencial continuar las investigaciones con los métodos modernos de diagnóstico bioquímicos, genéticos y moleculares, que permitan identificar otras causas, y que provean información para la aplicación de nuevas terapias. La precocidad del diagnóstico y el inicio del tratamiento representan factores importantes para el pronóstico. Los esfuerzos de detección temprana incluyen el beneficio del acceso a servicios que brinden intervenciones basadas en evidencia específicas para el autismo y su potencial, para mitigar o incluso prevenir los desafíos asociados con los síntomas del TEA, incluido el trastorno epiléptico, reducir los costos de atención y mejorar la calidad de vida de este grupo de pacientes.

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Participacion de los autores

Todos los autores contribuyeron a la concepción y revisión del estudio, definieron la metodología, analizaron los resultados, y desarrollaron la discusión. Todos los autores, asumieron la responsabilidad de la integridad del trabajo y dieron la aprobación final para su envío.

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Long-term effects of antenatal administration of corticosteroids. Where are we?

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Key words: corticosteroids, pregnancy; betamethasone; dexamethasone; delayed effects of medical treatment; neurologic manifestations; premature birth.

Abstract. There is increasing concern about the long-term effects of antenatal administration of corticosteroids, as some complications have been reported in neonates, adolescents and adults, which could be transmitted to subsequent generations, as it has been shown in animal and observational studies in humans. In this review, we summarize the current understanding of the long-term effects of antenatal corticosteroid administration and provide data to inform the preparation of guidelines. A literature search was performed in the PubMed database. A mechanism has been proposed as to how antenatal administration of corticosteroids could produce morbidity in neonatal neurodevelopment and lead to future diseases in adulthood. However, this hypothesis has not been proven in large randomized controlled trials. We summarize here the current data supporting and opposing the long-term effects of antenatal corticosteroid administration. Follow-up studies from randomized controlled trials have found no increased risk of neurologic impairment in children after exposure to a single course of antenatal corticosteroids. Observational and clinical trials of maternally administered antenatal corticosteroids show no evidence of increased disability on follow-up and describe associations rather than a proximate cause. Before 34 weeks of gestation, antenatal administration of corticosteroids in women at high risk for preterm birth appears to improve most neurodevelopmental outcomes. It is still recommended to administer a single course of corticosteroid treatment before preterm delivery.

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Efectos a largo plazo de la administración antenatal de corticosteroides. ¿Dónde estamos?

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Palabras clave: corticosteroides; embarazo; betametasona; dexametasona; efectos a largo plazo del tratamiento; manifestaciones neurológicas; parto pretérmino.

Resumen. Basándose en estudios en animales y observacionales en humanos, recientemente se han planteado una serie de dudas sobre los efectos a largo plazo de la administración antenatal de corticosteroides y se han notificado algunas complicaciones en neonatos, adolescentes y adultos; que incluso, podrían transmitirse a generaciones posteriores. Extenderla al periodo prematuro tardío, podría conducir a un aumento drástico del número de recién nacidos expuestos *in útero*. En esta revisión se resume el conocimiento actual de los efectos a largo plazo de la administración antenatal de corticosteroides y se aportan datos para la elaboración de guías para su uso. Para la metodología se realizó una búsqueda bibliográfica en la base de datos PubMed. Se ha propuesto un mecanismo para explicar cómo la administración antenatal de corticosteroides puede causar morbilidad en el neurodesarrollo neonatal y enfermedades programadas del adulto. Sin embargo, esta teoría no se ha demostrado en grandes pruebas controladas aleatorizadas. Aquí resumimos los efectos, a favor y en contra, a largo plazo de la administración antenatal de corticosteroides. La evidencia actual es inconsistente, los estudios de seguimiento de los ensayos controlados aleatorizados no han encontrado un mayor riesgo de deterioro neurológico en los niños, después de un solo curso antenatal de corticosteroides. Los ensayos observacionales y clínicos no muestran pruebas de aumento de discapacidad y describen asociaciones más que causas. Antes de las 34 semanas de embarazo, la administración antenatal de corticosteroides en mujeres con alto riesgo de parto pretérmino, parece mejorar la mayoría de resultados del neurodesarrollo. Antes del parto pretérmino, se recomienda un curso simple de tratamiento con corticosteroides.

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INTRODUCTION

Preterm birth (PB) is a significant public health problem in the United States, with 1 in 10 infants being born before term [1]. Antenatal administration of corticosteroids (ACS) is a worldwide standard of care for preventing and reducing perinatal morbidity and mortality (PM) [2-4]. To establish the standards for ACS, after more than five decades, two NIH Consensus Conferences were held in 1994 and 2000, and numerous pronouncements from important institutions and scientific societies worldwide have been made [5,6].

Recently, some complications have been reported in neonates, adolescents and adults, which could even be transmitted to subsequent generations, and concern has been raised about the long-term effects of

ACS [7]. Recent literature suggests that the benefits of ACS may extend to late-preterm and early-term infants as well. These expanded uses may expose populations to ACS at gestational ages that have been minimally evaluated for their efficacy or risks. Researchers continue to investigate the possible associations between ACS and long-term neurodevelopmental outcomes in exposed children, given the potential implications of the overuse of ACS [8].

The objective of this review is to describe the current status of the long-term effects of ACS, based on existing evidence.

Methods/design

We conducted a narrative review of the targeted literature focusing on the long-term effects of ACS. The PubMed database was searched for literature published up from January, 01 2015 to May 26, 2024, using the following keywords: "corticosteroid", "betamethasone", "dexamethasone", "antenatal betamethasone", "antenatal dexamethasone", "antenatal corticosteroids", "late preterm antenatal corticosteroids", "at term antenatal corticosteroids", "neurodevelopmental outcomes", "neonatal outcomes", and "long-term effects". We also included studies from other sources that we considered historical or relevant to the topic. We excluded studies that were not focused on the long-term effects of ACS or that focused on the description, guidelines, and other aspects of ACS.

Antenatal administration of corticosteroids

ACS has led to its demonstration of efficacy and safety, making it a worldwide standard of care for the prevention and reduction of fetal morbidity and PM (Tables 1 and 2) [1,2-4,5,6,9,10].

Additionally, there is evidence supporting the recommendation of a "rescue" course of corticosteroids if seven days have passed after the initial course [2,11-13].

ACS in late preterm pregnancy

Recently, ACS has been extended beyond 34 weeks of gestation (34.0 to 36.6 weeks of gestation) because studies demonstrate modest benefits from ACS for late preterm and term elective cesarean deliveries [3]. In 2016, a great multicenter, randomized trial [14] involved women with a singleton pregnancy at late preterm pregnancy who were at high risk for delivery. Participants received two injections of betamethasone phosphate/acetate or a matching placebo 24 hours apart. The primary outcome was a neonatal composite of treatment in the first 72 hours or stillbirth or neonatal death within 72 hours after delivery. Primary outcome occurred in 165 of 1427 infants (11.6%) in the betamethasone group and 202 of 1400 (14.4%) in the placebo group (relative risk in the betamethasone group, 0.80; 95% confidence interval [CI], 0.66 to 0.97; p= 0.02). In the betamethasone group, neonatal hypoglycemia was more common (24.0% vs. 15.0%; relative risk, 1.60; 95% CI, 1.37 to 1.87; p<0.001). ACS (betamethasone) significantly reduced the rate of neonatal respiratory complications in women at risk for late preterm delivery [14].

The Society for Maternal-Fetal Medicine (SMFM), in march 2016 [15], in the "Implementation of the use of ACS in the late PB period in women at risk for preterm delivery", recommended the treatment with a betamethasone phosphate/acetate single course, in women with late preterm gestation, with a singleton pregnancy, who are at high risk for PB within the next seven days, [15]. In October 2016, the American College of Obstetricians and Gynecologists (ACOG) [11], recommended the administration of betamethasone phosphate/acetate in late preterm gestations, in pregnant women who have not received a previous course of ACS and are at risk of PB within seven days [11].

Two trials of antenatal dexamethasone in preventing mortality and severe morbidity among late preterm newborns, in low-income

Table 1. Recommendations of scientific institutions on ACS in early preterm, late preterm and term gestation.

Gestation	SMFM [16]	ACOG [11,12]	RCOG [13,20]	FIGO [21]	WHO [17,22]	WAPM-PMF [10]	EGPC [19]
Early preterm	ACS prior to 34 weeks is standard practice for women at high risk for delivery in the next 7 days.	For pregnant women between 24 0/7 and 33 6/7 weeks at risk of PB within 7 days. Also for pregnant women starting at 23 0/7 at risk of PB within 7 days.	Should be offered to women between 24^{+0} and 34^{+6} weeks' gestation in whom imminent PB is anticipated	For whom PB is anticipated between 24 and 34 weeks, one course of ACS should ideally be offered, 18 to 72 before PB is expected.	ACS is recommended for women with a high likelihood of PB from 24 weeks to 34 weeks.	A single course of ACS should be administered bet-ween 24+0 and 33+6 weeks of gestation in women at high-risk of PB within the next 7 days.	ACS should be administered to women between 24 and 33weeks, when PB is anticipated in the next seven days.
Late preterm	To patients who meet the inclusion criteria of the Antenatal Late Preterm Steroids trial.	ACS may be considered in pregnant women between 34 0/7 and 36 6/7 weeks who are at risk of PB Within 7 days and who have not received a previous course of ACS.	In very late preterm women, ACS should be considered in light of the balance of risks and benefits.	ACS should not be offered routinely to women in whom late preterm birth between 34 and 36 weeks is anticipated.	ACS is not recommended for women undergoing planned caesarean section at 34 weeks 0 days to 36 weeks 6 days.	ACS is not routinely recommendded between 34+0 and 36+6 weeks in women at high-risk of PB within the next 7 days because of the current uncertainty regarding the benefit to risk ratio.	ACS between 34,0 and 34,6 weeks should only be offered to a few selected cases (Expert opinion). ACS between 35 and 36 weeks should be restricted to prospective randomized trials.
Term	–	–	For women undergoing planned caesarean birth between 37^{+0} and 38^{+6} weeks an informed discussion about the potential risks and benefits of ACS. Although ACS may reduce admission to the NICU for respiratory morbidity, it is uncertain if there is any reduction in RDS, transient tachypnoea or NICU admission, and may result in hypoglycaemia and potential developmental delay morbidity.	ACS should not be given routinely before cesarean delivery at term.	–	ACS is not routinely recommended before scheduled cesarean section at term because of the current uncertainty regarding the benefit to risk ratio. In the absence of other indications, a scheduled cesarean section should not be performed before 39+0 weeks.	ACS in pregnancies beyond 37weeks is not indicated, even for scheduled cesarean delivery.

SMFM: society of maternal fetal medicine ACOG: American college of obstetricians and gynecologists RCOG: royal college of obstetricians and gynecologists FIGO: federation international of obstetrics and gynecology WHO: world health organization WAPM-PMF: world association of perinatal medicine-perinatal medicine foundation EGPC: European guide of perinatal care ACS: antenatal administration of corticosteroids PB: preterm birth.

Table 2. Benefits and risk of ACS.

– ACS before 34 weeks presents an undeniable benefit for the unborn child if born prematurely.
– Identifying patients who will actually deliver within 7 days following the treatment is difficult and further studies need to be conducted.
– After 34 weeks of gestation the benefit/risk balance is against ACS.
– Studies should be conducted on reducing ACS.

ACS: antenatal administration of corticosteroids.

countries, did not show adverse childhood neurodevelopmental outcomes and proved safe and efficacious [16,17]. In the ALPS follow-up study of a Randomized Controlled Trial (RCT) 16, ACS initially showed improvement in short-term neonatal respiratory outcomes at age six years or older, without adverse childhood neurodevelopmental outcomes, but with an increased rate of hypoglycemia [16]. In a multicenter, two-arm, parallel, double-blind, placebo-controlled, randomized trial[17]; antenatal dexamethasone for late preterm birth did not result in a reduction in neonatal death, stillbirth, or severe neonatal respiratory distress [17].

Currently, there is no agreement on the use of ACS in the late preterm period, as the SMFM [18] and ACOG [12] recommend its use in the United States, whereas many institutions and scientific societies worldwide do not [10,13,19-22].

The expanded use of ACS in late preterm pregnancy

Literature suggests that the benefits of ACS may extend to late-preterm and early-term infants as well [2,11,12,14,18]. This expanded use exposes populations to ACS at gestational ages that have been minimally evaluated for efficacy or risk. Extending the use of ACS to the late preterm period may lead to a dramatic increase in the number of infants exposed *in utero* to ACS (nearly 10% of fetuses) and an even greater increase in the proportion of fetuses exposed to ACS, which will eventually be born at term. As treatments expand, benefits decrease and risks increase[3,23-27].

ACS in a term pregnancy

In 2005, the multicenter RCT study ASTECS (Antenatal Steroids for Term Elective Cesarean Section) included 998 women with term pregnancies, of whom 503 received a single course of betamethasone phosphate/acetate in the 48 hours preceding a cesarean section. The primary outcome was admission to the special care baby unit with respiratory distress. Of the 35 children admitted to the special baby units with respiratory distress, 24 babies were in the control group and 11 in the intervention group, a statistically significant difference ($p = 0.02$). The incidence of admission with respiratory distress in the control group was 0.051, and in the treatment group, was 0.024 (relative risk 0.46, 95% confidence interval 0.23 to 0.93). In this study, both ACS and delaying delivery until 39 weeks reduce admissions to special care baby units with respiratory distress after elective cesarean section at term[28]. In 2009, a Cochrane systematic review [29,] concluded that results from the single trial are promising, but more studies with larger samples were needed.

Additionally, more data and a longer follow-up would be needed to assess potential harms and complications. In October 2010, the Royal College of Obstetricians and Gynecologists of the United Kingdom [13] established that there is a lack of evidence available regarding the safety of ACS in babies born after 36+0 weeks of gestation. Elective lower-segment cesarean section should not typically be performed until 39+0 weeks of gestation, rather than the ACS [13].

ACS and long-term effects

The increased use of ACS during the late prenatal period and prior to cesarean sections at term, due to their proven efficacy and safety, has resulted in a significant rise in the number of infants exposed to ACS in utero. Approximately three-fourths of all PB occur in the late preterm period [16]. Currently, almost 10% of fetuses are exposed *in utero* to ACS, and >50% of women given ACS for presumed PB will not deliver within the optimal exposure window of seven days [3,7,24,30]. The net effect is that a substantial fraction of the delivery population will be exposed to ACS, and more fetuses exposed to ACS are delivered at term (most recipients deliver after 35 weeks' gestation and 44% to 50% deliver at term) [3,7,24].

The New Zealand Group followed children who received betamethasone *in utero* during the study of Liggins study [4] for many years, 2005-2007, establishing that [2]: 1. ACS with a single course of betamethasone do not alter psychological, pulmonary and cardiovascular functions at 30-31 years of follow-up. 2. Adults who were born moderately preterm have increased blood pressure and insulin resistance at 30 years of age; 3. PB, and not poor fetal growth, is the primary determinant of this association; 4. Obstetricians should continue to use a *single course* of ACS for the prevention of neonatal respiratory distress syndrome (RDS).

Based on animal and observational studies in humans, several concerns have recently been raised about the long-term effects of ACS, and some complications have been reported in neonates, adolescents, and adults, which could even be transmitted to subsequent generations [7,18]. Although the long-term risks associated with ACS use (such as neonatal neurodevelopmental issues and adult program diseases) remain uncertain, several observations have been reported on the likely long-term effects of ACS. Late effects of ACS suggest caution for the expanded use of ACS beyond at-risk pregnancies at 24-34 weeks [2,24].

ACS and the hypothalamic-pituitary-adrenal axis

In animal studies, exposure to exogenous ACS has been associated with a delay in brain growth and development, as well as increased activity and persistent changes in the hypothalamic-pituitary-adrenal (HPA) axis and endogenous corticosteroid production. At the molecular level, transcriptional changes occur in metabolic and growth pathways. Epigenetic mechanisms participate in transgenerational inheritance, not genomic[7,25]. Exposures that alter the methylation status of the 11β-hydroxysteroid dehydrogenase type 2 enzyme in the placenta can lead to transcriptional repression of the gene, resulting in the fetus being exposed to higher levels of cortisol. Such an adaptation to an intrauterine insult is thought to be beneficial in the short term and is linked to an increased chance of offspring survival to reproductive age. In adults, activation of the HPA axis has been linked to increased likelihood of cardiovascular risk factors comprising the metabolic syndrome, including higher glucose, blood pressure, and triglycerides, as well as ischemic heart disease, cognitive decline, and depression in later life[25]. It remains to be determined whether the HPA axis hyperactivity contributes to such effects [7,25,26].

Evidence on the long-term effects of ACS

Let us now review some recent animal and human evidence on the long-term effects of ACS (Table 3).

Animal studies

In utero exposure to single doses of exogenous corticosteroids may affect fetal brain development and neurological outcomes, as suggested by some animal studies. The hypothesis proposed by Seckl in 2004 [31] was recognized in an editorial by Reynolds and Seckl [25], indicating that rats and lambs exposed to an adverse in utero environment during development can experience long-term effects on physiology and an increased risk of adult disease. Overall, the

Table 3. Evidences on the long-term effects.

Authors	Year	Type of evidence	Results/Conclusions/Comments
			Evidence in animals
Reynolds, Seckl [25]	2012	Editorial	Recognize the hypothesis of Seckl [31], in 2004.
Seckl [31]	2004	Review	Hypothesized that prenatal exposure to excess glucocorticoids or stress might represent a mechanism linking fetal growth with adult pathophysiology. The data suggest that both pharmacological and physiological exposure prenatally to excess glucocorticoids, programmes cardiovascular, metabolic and neuroendocrine disorders in adult life. It was published in 2004, i.e. 19 years ago. It was based on a majority of animal studies. Most of the cited animal studies, in which weight reduction was obtained, were conducted between 1978-2001, and used multiple courses of ACS, characterized because they do not enhance the beneficial effects of single courses and because produce fetal injury. Multiple courses of ACS are not recommended since 2000 [6].
Jobe, Goldenberg [3]	2018	Clinical opinion	Noted that cardiovascular and metabolic abnormalities have been identified in large animal models and cohorts of children exposed to ACS, that are consistent with fetal programming for adult diseases.
			Evidence in humans
			A single course of ACS
Smolder et al. [33]	1990	RCT	ACS does not alter lung function or the prevalence of wheeze and asthma at age 30. Found no difference in neurologic and ophthalmologic examination results.
Dalziel et al., cited by [2]	2006	Longitudinal by 30 years	ACS of a single course of betamethasone, found no increased risk of impairment in cognitive functioning, working memory, attention, psychiatric morbidity, or health-related quality of life.
Stutchfield et al. [34]	2013	Multicenter RCT (ASTECS trial),	No difference in behavioral, cognitive, or developmental outcomes among children aged 8-15 years whose mothers received a single course of ACS before elective cesarean delivery at 37-38 weeks of gestation.
Sotiriadis et al. [35]	2015	Systematic review	A single course of ACS in women at high risk for PB appears to improve most neurodevelopmental outcomes in offspring born before 34 weeks of gestation.
Alexander et al. [36]	2016	Cross-sectional study	A single course of ACS does not aggravate long-term cognitive deficits. Our data indicated that conditions related to a threatening PB rather than ACS per se, were associated with long-term decreases in the child's intelligence.
Melamed et al. [27]	2019	Retrospective cohort study	Children exposed to ACS were more likely to have suspected neurocognitive disorders.
Raikkönen et al. [8]	2020	Retrospective cohort study	Siblings exposed to ACS were more likely to exhibit any childhood mental or behavioral disorder.
Ninan et al. [37]	2022	Systematic review	A single course of ACS, was associated with a significant decrease in the adjusted odds of neurodevelopmental impairment in children with extremely preterm birth..
Hutcheon et al. [38]	2022	Regression discontinuity design	Found little evidence that children with higher probability of exposure to ACS have higher rates of ADHD prescriptions in childhood, supporting the safety of ACS for this neurodevelopmental outcome.
			Multiple course of ACS
Asztalos et al. [42]	2014	Secondary analysis of the RCT MACS (MACS-5 trial)	Children born ≥ 37 weeks and exposed to multiple ACS therapy may have an increased risk of neurodevelopmental/neurosensory impairment by 5 years of age.

ACS: Antenatal administration of corticosteroids RCT: Randomized controlled trials ASTECS: Antenatal betamethasone for term caesarean section PB: Preterm birth ADHD: Attention deficit/hyperactivity disorder MACS: Multiple antenatal courses of steroids.

data suggest that both pharmacological and physiological exposure prenatally to excess glucocorticoids can lead to cardiovascular, metabolic and neuroendocrine disorders in adult life [31].

Concerning the Seckl's study [31], it should be noted that: 1. It was published in 2004, i.e. 20 years ago. 2. It was based on a majority of animal studies. 3. Most of the cited animal studies, in which weight reduction was obtained used multiple courses of ACS (defined as the use of ACS multiple courses: three, four, five, six or more courses), characterized because they do not enhance the beneficial effects of single courses and because produce fetal injuries and were conducted between 1978 and 2001 [2]. For these reasons, they have not been recommended since 2000. The four extensive studies worldwide, using multiple ACS courses, reported fetal injuries (TEAMS, NIH, ACTORDS, and MACS) [2,6]. On multiple courses, in the section "Human Clinical Observations," Seckl[31] notes that a single course of ACS is associated with a significant reduction in the incidence of intraventricular hemorrhage and a trend toward fewer neurodevelopmental disabilities. However, a survey by the British Obstetric Departments showed that 98% of participants were prescribing multiple courses of ACS. There is little evidence of the safety and efficacy of such a regime. Recent overviews suggest that there is no evidence of additional benefits from multiple courses of glucocorticoid therapy during pregnancy, but that clear conclusions are prevented by the lack of prospective RCTs and by variations in the protocols employed. ACS has also been linked with higher blood pressure in adolescence and subtle effects on neurological function, including reduced visual closure and visual memory. In 2018, Jobe and Goldenberg [3] noted in a clinical opinion article that cardiovascular and metabolic abnormalities have been identified in large animal models and cohorts of children exposed to ACS, which are consistent with fetal programming for adult diseases [3]. After 34 weeks of gestation, when rapid brain growth occurs, theoretically, these effects could be more pronounced [32].

Human studies:

A single course of ACS

In children born after exposure to a single course of ACS, follow-up studies from RCTs of ACS have found no increased risk of neurologic impairment. Their precise assessment of neurodevelopmental outcomes was a strength of these studies [16]. However, findings are mixed, in observational studies.

No differences between the corticoid and the placebo groups were found in 1990, in one RCT of betamethasone, when Smolder et al. [33] studied potential side effects of ACS to prevent neonatal RDS, in 10- to 12-year-old children (N: 84). The lung function as assessed by the presence of wheeze and asthma at age 30, was not altered by ACS. This study found no difference in neurologic and ophthalmologic examination results [33]. These findings were similar to those of a longitudinal study conducted in 2006, which followed 534 30-year-old adults[33]. ACS by a single course of betamethasone did not increase the risk of impairment in lung function, cognitive functioning, working memory, attention, psychiatric morbidity, or health-related quality of life between the betamethasone and placebo groups [2]. In a questionnaire-based follow-up of a multicenter RCT (ASTECS Trial), Stutchfield et al. [34] demonstrated no difference in behavioral, cognitive, or developmental outcomes among children aged 8 to 15 years whose mothers received a single course of ACS before elective cesarean at 37 to 38 weeks of gestation [34]. In 2015, Sotiriadis et al. [35], concluded that a single course of ACS in women at high risk for PB appears to improve most neurodevelopmental outcomes in offspring born before 34 weeks of gestation as showed by reduced risk for cerebral palsy (seven studies; treated: 390 of 5,199, untreated: 146 of 1,379; RR 0.678, 95% confidence interval [CI] 0.564-0.815), psycho-

motor development index less than 70 (two studies; treated: 783 of 3,049, untreated: 258 of 969; RR 0.829, 95% CI 0.737-0.933), and severe disability (five studies; treated: 1,567 of 4,840, untreated: 475 of 1,211; RR 0.787, 95% CI 0.729-0.850); in a systematic review, included RCT and non RCT, reporting on the neurodevelopmental outcomes of children whose mothers were administered a single course of ACS for threatened PB [35]. In a cross-sectional study of a mixed-sex cohort of 222 term-born children (aged 6-11 years), Alexander et al. [36] concluded that their data indicated that ACS does not aggravate long-term cognitive deficits [36].

In 2019, a retrospective cohort study (2006-2011), by Melamed et al. [27], examined outcomes at five years of age in 5432 children exposed to ACS compared with 523,782 children not exposed. Children exposed to ACS were more likely to have suspected neurocognitive disorders (exposure to ACS vs no exposure: 25.8% vs 21.6%; p<0.001; adjusted hazard ratio [aHR] 1.16, 95% CI 1.10 to 1.21) [27]. In 2020, Raikkönen et al. [8] conducted a population-based retrospective cohort study using nationwide registries of all singleton live births in Finland that survived until one year, along with a within-sib-pair comparison among term siblings. They found that siblings exposed to ACS were more likely to exhibit any childhood mental or behavioral disorder and concluded that ACS was significantly associated with mental and behavioral disorders in term-born children (12.01% among those exposed to ACS vs 6.45% among those not exposed; absolute difference, 5.56% [95% CI, 5.04%-6.19%]; p<.001) [8]. In 2022, in another systematic review and meta-analysis of 30 studies involving more than 1.25 million children, Ninan et al. [37] noted that exposure to a single course of ACS was associated with a significant decrease in the adjusted odds of neurodevelopmental impairment in children with extremely high PB. ACS exposure was associated with increased adjusted risks of neurocognitive and/or psychological impairment in children with late-preterm and full-term birth [37]. Furthermore, in 2022, Hutcheon et al. [38], found little evidence that children with a higher probability of exposure to ACS have higher rates of attention-deficit/hyperactivity disorder (ADHD) prescriptions in childhood, supporting the safety of ACS for this neurodevelopmental outcome. They used a regression discontinuity design, over a median follow-up period of nine years, 892 (5.5%) children had one or more dispensations of ADHD [38]. In 2022, Sarid et al. [39], synthesized the association between ACS exposure and the risk of preterm birth and brain development in infants ultimately born at late preterm and term. Their protocol included 27 observational studies (12 retrospective cohort studies, 11 prospective cohort studies and four cross-sectional studies). In 14 studies, a single course of ACS was administered; in two studies, two or more courses of ACS were administered. In seven studies, the number of courses of ACS varied among study participants and in four studies, the regimen was not specified. The most common adverse outcomes were reduced neonatal head circumference, structural cortical differences on magnetic resonance images, increased prevalence of psychiatric problems, and increased risk of neurodevelopmental delays, in ACS-exposed late preterm and term infants. Further research, such as preterm labor, maternal stress, and the number of ACS courses, is needed to establish better the long-term neurological effects of ACS on late preterm and term infants, given that the existing research was at serious risk for bias [39]. In 2023, Yao et al. [40], investigated the associations between ACS and serious infections in children during the first 3, 6, and 12 months of life. This nationwide cohort study found that children exposed to one course of ACS were significantly more likely to have an increased risk of serious infection during the first 12 months of life. The study cohort consisted of 1,960,545 singleton children: 45,232 children were exposed to one course of ACS, and 1,915,313 children were not ex-

posed. The adjusted hazard ratios for overall serious infections, sepsis, pneumonia, and acute gastroenteritis, among children exposed to ACS, were significantly higher than those not exposed. The adjusted hazard ratios for overall serious infection ($p<0.001$), sepsis ($p = 0.02$), pneumonia ($p<0.001$), and acute gastroenteritis ($p<0.001$) were significantly higher from birth to 12 months of life [40]. In 2023, Weiss et al. [41] determined if ACS exposure was associated with infant cortisol levels. One hundred eighty-one women and their infants participated in the study. Infants whose mothers received ACS had significantly lower resting-state (B = -2.47, CI: -3.691; to 0.0484) and post-stressor (B = 0.51, CI: -4.283, to -0.4276) cortisol levels across the first year of life than infants whose mothers did not receive ACS. Results indicate a state of dampened HPA activation and cortisol hypo-arousal that persists across the first year of life among infants who were exposed to ACS in utero. They concluded that further research is needed to examine the mechanisms responsible for any alterations that occur during the development of the fetal HPA axis, including epigenetic and biochemical factors [41].

Multiple, repeat or weekly courses of ACS:

In 2000, studies examining multiple courses of ACS, were reserved for patients enrolled in clinical trials [2,6]. However, we will point out that after multiple courses of ACS, different outcomes have been observed. In 2014, in the trial MACS-5, Asztalos et al. [42] concluded that children born ≥ 37 weeks and exposed to multiple ACS therapy may have an increased risk of neurodevelopmental/neurosensory impairment by five years of age [42]; they studied the association between gestational age at birth, multiple courses of ACS and outcomes at five years, In the previously cited 2022, Sarid et al.´s trial [39], two or more courses of ACS were administered.

Limitations of previous studies

Some limitations have been identified in previous studies (Table 4) [18,24,42,43]. Data from RCT on the long-term effects of ACS on the fetal brain are limited [22].

Table 4. Limitations to evidences on the long-term effects of ACS [18,24,42,43].

- Many of these studies either were from small samples or used population-based registry data, from which the severity of illness may be difficult to discern.
- Given the retrospective, observational study designs, the authors did not account for the admission or delivery indication or other antenatal complications (such as fetal growth restriction, oligohydramnios, infections during pregnancy, red blood cell alloimmunization, hydrops, or congenital anomalies) that may substantially influence long-term neurologic outcomes.
- Previous studies have suggested that it is not, in fact, the ACS* itself but rather the condition leading to a threat of PB† that is associated with neurologic impairment in exposed children.
- Information regarding steroid type, dosing, frequency, and timing of exposure in most studies is unknown.
- There is a potential bias related to differential referral or diagnosis of subjects exposed to ACS with abnormal development
- The lack of standardized evaluations by trained professionals blinded to exposure status, along with the absence of defined diagnostic criteria, leaves room for bias and subjectivity in diagnosis.

ACS: Antenatal administration of corticosteroids PB: Preterm birth.

To answer the question of where we stand, we summarize the current data for and against the long-term effects of ACS.

Evidence for and against the long-term effects of ACS

In recent years, there has been much evidence for and against the long-term effects of ACS (Table 5) [2,3,7-9,18,23-27,29,35-37,42,43].

Summary and conclusions

Animal studies have found that ACS affect many organs. Observational and clinical trials of maternal ACS show no evidence of increased disability on follow-up and describe associations rather than a proximate cause.

ACS in women at high risk for PB appears to improve most neurodevelopmental

Table 5. Evidence for and against long-term effects of ACS [2,3,7-9,18,23-27,29,35-37,42,43]

FOR

– Several concerns have been raised on the potential long-term effects of ACS* on cerebral function and neonatal growth.
– It is now well recognized that exposure to an adverse in utero environment during development has long-term effects on physiology and later risk of adult disease.
– Large observational studies have reported reduced neonatal weight and length in children exposed to ACS.
– Cardiovascular and metabolic abnormalities have been identified in large animal models and cohorts of children exposed to ACS that are consistent with fetal programming for adult diseases.
– There are observational data linking exposure to multiple courses of ACS to increased rates of aggressive, destructive, distractible and hyperkinetic behavior at both ages of 3 and 6 years.
– Siblings exposed to ACS were more likely to exhibit any childhood mental or behavioral disorder.
– The RCT data also do not strongly support the optimal interval from ACS to delivery of 1-7 days.
– Epidemiology-based studies using large cohorts with >85% of at-risk pregnancies treated with ACS, probably overestimate the benefits of ACS.
– Although most of the prematurity-associated mortality is in low-resource environments, the efficacy and safety of ACS in those environments remain to be evaluated.

AGAINST

– The long-term outcomes in human children are not well understood. Animal studies have found that ACS affect many organs across multiple stages of life.
– Data from RCT on the long-term effects of ACS on the fetal brain are limited. Another important factor is that many of the included infants were born prematurely and it is likely that the immediate benefits of ACS compensate for the potential long-term adverse effects of ACS.
– Observational reports describe associations rather than proximate cause and are subject to bias from undetected and undetectable confounders.
– These high evidence level randomized data will allow us to reframe the late preterm ACS discussion around the uncertain risk of developmental delay.
– ACS-exposed children born extremely preterm had a markedly lower risk of neurodevelopmental impairment and/or psychological outcomes when compared with unexposed children.
– ACS in women at high risk for PB appears to improve most neurodevelopmental outcomes in offspring born before 34 weeks of gestation and is significantly associated with reduced mortality before 3 years of age in very low birth weight infants with chorioamnionitis (do not alter neurodevelopmental outcomes).
– Other studies either found no difference in neurodevelopmental outcomes or attributed the increased observed risk for adverse neurologic outcomes among term-born children to receiving multiple courses (rather than a single course) of ACS or to the underlying condition threatening preterm delivery rather than the steroid exposure itself.
– The short-term benefits of ACS for high-risk pregnancies in high-resource environments certainly justify ACS. Single course ACS treatment before preterm delivery must still be recommended as life-saving and cost effective intervention. Late effects of ACS suggest caution for the expanded use of ACS beyond at-risk pregnancies at 24-34 weeks
– Multiple ACS courses are not recommended since 2000.

ACS: Antenatal administration of corticosteroids RCT: Randomized controlled trials PB: Preterm birth.

outcomes before 34 weeks of gestation, and reduces mortality before three years of age in very low birth weight infants with chorioamnionitis.

Other studies either found no difference in neurodevelopmental outcomes or attributed the increased observed risk for adverse neurologic outcomes among term-born children to receiving multiple courses of ACS or to the underlying condition threatening PB rather than the steroid exposure itself.

On the long-term effects of ACS on the fetal brain, there are limited data from RCTs. The interpretation of available RCT data is further hampered by both the lack of an unexposed control group and insufficient power to detect significant differences in rare events. Additionally, many of the included infants were born prematurely, which is another important factor.

In high-resource environments, the short-term benefits of ACS for high-risk pregnancies certainly justify ACS. Single-course ACS treatment before preterm delivery must still be recommended as a life-saving and cost-effective intervention.

Cautioning against the expanded use of ACS beyond at-risk pregnancies at 24-34 weeks suggests late effects of ACS.

Multiple ACS courses are not recommended since 2000.

The long-term outcomes in human children are not well understood. Therefore, the possible association between ACS and long-term neurodevelopmental outcomes in exposed children should be further investigated. Specifically, longitudinal studies and large RCTs are needed to provide more insight into this topic.

Statement of ethics

This study was approved by the University of Zulia Ethics Committee (number 21-2024) on March 21, 2024. It adhered to the laws and national ethical guidelines of Venezuela, as well as the COPE guidelines and the Declaration of Helsinki.

Conflict of interest

The authors declare that they have no conflicts of interest.

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CBP contributed to the study conception. CBP, LBS, ERV and PVDG contributed to the design, researched literature, analized it and design the protocol, manuscript revision and approved final version.

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