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ORIGINAL ARTICLE

# Hypoglycemic effect of NeuroEPO in diabetic and non-diabetic rats

## Efecto hipoglicemiante de la NeuroEPO en ratas con y sin diabetes mellitus

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## ABSTRACT

**Introduction:** NeuroEPO is a non-hematopoietic variant of human recombinant erythropoietin, which may have a hypoglycemic effect.

**Objectives:** To evaluate the influence of NeuroEPO on glycemia in diabetic and non-diabetic rats.

**Material and Methods:** The experiments were conducted in Wistar rats with streptozotocin-induced diabetes with and without insulin treatment, and in non-diabetic rats with glucose overload. In each experiment, one group received a subcutaneous injection of NeuroEPO (0.5 mg/kg) and the other group received a vehicle. Glycemia was determined in 120 min. Comparisons were made using one-and two-way analysis of variance, followed by the Bonferroni test. The differences were considered significant with p values < 0,05.

**Results:** In diabetic rats without insulin treatment, glycemic levels decreased significantly in the group that received NeuroEPO. In nondiabetic rats that received NeuroEPO and a glucose overload, glycemia was similar to that in the control group. In diabetic rats that received NeuroEPO and insulin, the glycemia reduction was greater than in the group that only received insulin.

**Conclusions:** NeuroEPO has a hypoglycemic effect in diabetic rats due to an insulinotropic mechanism that shows synergism with insulin in the treatment of hyperglycemia. However, NeuroEPO does not influence the glucose tolerance in non-diabetic rats, at least immediately. It is necessary to delve into the mechanisms by which NeuroEPO can reduce hyperglycemia and the influence of this substance under conditions of normoglycemia.

**Keywords:**
diabetes *mellitus*; erythropoietin and cytoprotection; glucose tolerance test; insulin tolerance test; NeuroEPO; streptozotocin.

## RESUMEN

Introducción: La NeuroEPO es una variante no-hematopoyética de la eritropoyetina recombinante humana, que pudiera tener efecto hipoglicemiante.

Objetivo: Evaluar la influencia de la NeuroEPO sobre la glicemia de ratas con diabetes *mellitus* y ratas no-diabéticas.

Material y Métodos: Se realizaron experimentos en ratas Wistar con diabetes inducida por estreptozotocina, con y sin tratamiento con insulina, y en ratas no-diabéticas con una sobrecarga de glucosa. En cada experimento, un grupo recibió una inyección subcutánea de NeuroEPO (0,5 mg/kg) y otro el vehículo, y se determinó la glicemia durante 120 minutos. Se realizaron comparaciones mediante análisis de varianza de una y dos vías, seguidas por la prueba de Bonferroni. Las diferencias se consideraron significativas con valores de p < 0,05.

Resultados: En las ratas diabéticas sin tratamiento con insulina, los niveles de glicemia del grupo con NeuroEPO disminuyeron de forma significativa. En las ratas no-diabéticas que recibieron NeuroEPO y una sobrecarga de glucosa, la glicemia fue similar al grupo control. En las ratas diabéticas que recibieron NeuroEPO e insulina la reducción de la glicemia fue mayor que en el grupo que solo recibió insulina.

Conclusiones: La NeuroEPO tiene un efecto hipoglicemiante en ratas diabéticas, por un mecanismo insulinotrópico que muestra sinergismo con la insulina en el tratamiento de la hiperglicemia. Sin embargo, la NeuroEPO no influye en la tolerancia a la glucosa de ratas no-diabéticas, al menos de forma inmediata. Es necesario profundizar en los mecanismos mediante los cuales la NeuroEPO puede reducir la hiperglicemia, y la influencia de esta sustancia en condiciones de normoglicemia.

Palabras Claves:
Eritropoyetina y citoprotección, NeuroEPO, diabetes *mellitus,* estreptozotocina, prueba de tolerancia a la glucosa, prueba de tolerancia a la insulina.

## INTRODUCTION

*Diabetes mellitus* (diabetes) is a heterogeneous syndrome characterized by chronic hyperglycemia and disorders in the metabolism of carbohydrates, lipids, and proteins as a result of an absolute or relative deficit of insulin secretion, or resistance to the action of this hormone. Its origin is multifactorial and its causes include genetic, environmental, immunological, and viral factors.[1]

Diabetes statistics indicate that it is a major health problem in the world. The notable increase in morbidity and mortality from diabetes has determined that it is currently recognized as a true pandemic.[1] Statistics in Cuba place diabetes among the top 10 causes of death,[2] as it happens worldwide.

To date, there is no cure for diabetes, and the most effective tool to decrease complications continues to be strict metabolic control. However, it is difficult to achieve stable metabolic control in diabetic patients, so it is necessary to evaluate new treatment strategies.[3]

Erythropoietin (EPO) is a glycoprotein whose primary function is to stimulate erythropoiesis, but in recent years its distribution and that of its receptor in non-hematopoietic tissues have been demonstrated,[4] suggesting that it is involved in other physiological activities. The non-hematopoietic effect of EPO that has been well documented is the protection and repair of damaged tissues to which its angiogenic, anti-apoptotic, anti-inflammatory, neurotrophic, and antioxidant actions contribute.[4]

The cytoprotective effect of EPO is mediated by a receptor other than erythropoiesis linked (EPOR), known as TPR (tissue-protective receptor*),* which is expressed mainly in injured or metabolically stressed tissue; its expression precedes the local increase in tissue production of EPO, so the application of exogenous EPO could accelerate innate cytoprotective mechanisms.[5]

Human recombinant EPO (rhuEPO) has been widely used in the treatment of several types of anemias,[4] but there are multiple studies on its influence on the recovery of damaged tissues in organs such as the brain,[6,7,8] the heart,[9,10] the lungs,[11] and skeletal muscle.[12]

In addition, there is evidence that rhuEPO reduces glycemia in conditions of hyperglycemia, so research on its potential cytoprotective role in diabetes has increased.[13,14,15,16,17,18]

Moreover, activation of EPO receptors in non-hematopoietic tissues is known to require higher EPO concentrations than those needed to stimulate erythropoiesis. Therefore, the use of high doses of rhuEPO produces adverse effects associated with the stimulation of hematopoietic and procoagulant pathways, especially hypertension and venous thromboembolism.[4] For this reason, the clinical use of rhuEPO in tissue protection has been limited and derivatives have been developed that enhance cytoprotective effects, but without hematopoietic actions.[4.5]

At the Center for Molecular Immunology of Cuba, during the production of rhuEPO, a variant with a low sialic acid content, known as NeuroEPO, is obtained.[19] The use of a nasal formulation containing NeuroEPO as an active ingredient has shown neuroprotection in models such as cerebral ischemia[19,20,21] and Alzheimer's disease,[19,22,23] without adverse effects at the hematological level.[19.24]

Considering the evidence that rhuEPO reduces glycemia in diabetes *mellitus*, the **objective** of this work is to evaluate the influence of NeuroEPO on glycemia in diabetic and non-diabetic rats.

## MATERIAL AND METHODS

An experimental study was carried out in Wistar rats (**Figure 1**), obtained from the National Center for Production of Laboratory Animals (CENPALAB) in Cuba, following the established ethical precepts.[25] The experiments were carried out in the laboratories of the Department of Biochemistry of the Institute of Basic and Preclinical Sciences "Victoria de Girón", between 2019 and 2020. In each group, a number of animals similar to that reported in previous research in which glucose homeostasis in diabetic rats was evaluated.[13,15,16,17,26]

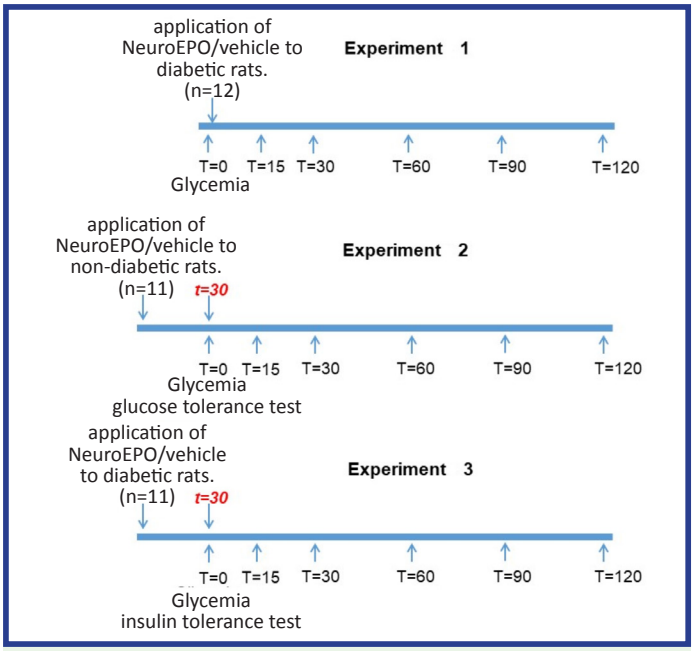


**Figura 1-** General study design

Three experiments were performed on Wistar rats with 6 hours of fasting starting in the morning. T: time (minutes) in which glycemia was determined; PTG, glucose tolerance test; ITP: insulin tolerance test. In experiments 2 and 3, the first glycemia (T0) was performed within 30 minutes (t=30) of the administration of NeuroEPO or vehicle.

### Animals and environmental conditions

Female, fertile and virgin rats were used, with an initial weight of 200 g ± 20 g; they were kept in independent boxes, with constant cycles of light and obscurity, at a temperature of 21 - 23 ºC, and with free access to standard food[26] (CENPALAB) and filtered water. The animals were kept for a week in adaptation to the environment before starting the procedures.

### Induction of diabetes

Diabetes was induced by intraperitoneal injection of streptozotocin (SIGMA), 65 mg/kg in 200 µL of sodium citrate buffer 0.1 M pH 4.5; one week later glycemia was determined and rats with glycemia greater than 11 mM were considered diabetic.[26] Once diabetes was diagnosed, the rats were randomly distributed into the study groups.

### Preparations and route of administration

NeuroEPO and vehicle (supplied by the Center for Molecular Immunology of Cuba) were used, which were administered to the corresponding groups subcutaneously, in the dorsal region of the animals. The dose of NeuroEPO evaluated was 0.5 mg/kg and the same volume of vehicle as NeuroEPO was administered.

### Determination of glycemia

Before each experiment, the rats were kept on a six-hour fast starting in the morning.[27] The levels of glycaemia were determined using a SUMA glucometer and its biosensors in blood obtained from a cut at the tip of the animal's tail. The results and the percentage of the initial glycemia (%) were analyzed;[26] the initial glycemia was considered to be 100 % which corresponded to the glycemia at time 0 of each experiment.

### Experiment 1

In order to evaluate the influence of NeuroEPO on the glycemia of diabetic rats, two groups of 12 rats each were used:

- D-NeuroEPO: diabetic rats that received a single application of the NeuroEPO.

- D-vehicle: diabetic rats that received a unique application of the vehicle.

Glycemia was determined before administering the NeuroEPO or the vehicle (time 0), and at 15, 30, 60, 90, and 120 minutes after the corresponding substance was supplied.

#### Experiment 2

In order to evaluate the influence of NeuroEPO on glucose tolerance in non-diabetic rats, two groups of 11 rats each were used:

- ND-NeuroEPO: Healthy rats that received a single application of the NeuroEPO, followed by a glucose tolerance test.

- ND-vehicle: healthy rats that received a unique application of the vehicle, followed by a glucose tolerance test.

Within 30 minutes of the application of the NeuroEPO or the vehicle, glycemia was determined (time 0) and a glucose tolerance test was performed; the test consisted of administering a glucose solution 2 g/kg orally, by tube, and then determining glycemia at 15, 30, 60, 90 and 120 minutes.[27]

#### Experiment 3

In order to evaluate the influence of the combination of NeuroEPO and insulin on the glycemia of diabetic rats, two groups of 11 rats each were used:

- D-NeuroEPO-insulin: Diabetic rats that received a single application of the NeuroEPO, followed by an insulin tolerance test.

- D-vehicle-insulin: diabetic rats that received a single application of the vehicle, followed by an insulin tolerance test.

Within 30 minutes of the application of the NeuroEPO or the vehicle, glycemia was determined (time 0) and an insulin tolerance test was performed; the test consisted of administering 0.75 IU/kg of insulin subcutaneously in the dorsal area of the rat, and then determining glycemia at 15, 30, 60, 90 and 120 minutes.[27]

#### Statistical processing

The Program GraphPad Prism, version 5.01, was used. Comparisons were made using one-way and two-way analysis of variance (ANOVA), followed by the Bonferroni multiple comparison test. The differences were considered significant with values of $p< 0.05$. The results were expressed as values of the mean and the standard error of the mean.

The primary data of this research are deposited in Mendeley Data as a principle of open access to information.[28]

The research is part of a national project of the Ministry of Public Health of Cuba (code 1901086).

## RESULTS

In experiment 1, in which NeuroEPO or the vehicle was administered to diabetic rats without insulin treatment, a reduction in glycemia was observed in the group that received the NeuroEPO, which became significant at 120 minutes compared to baseline glycemia and that of the group with the vehicle (**Figure 2 A**).

When analyzing the results of experiment 2, in which the NeuroEPO or vehicle was administered to non-diabetic rats and then a glucose tolerance test was performed, similar glycemic levels were found in both groups (**Figure 2 B**).

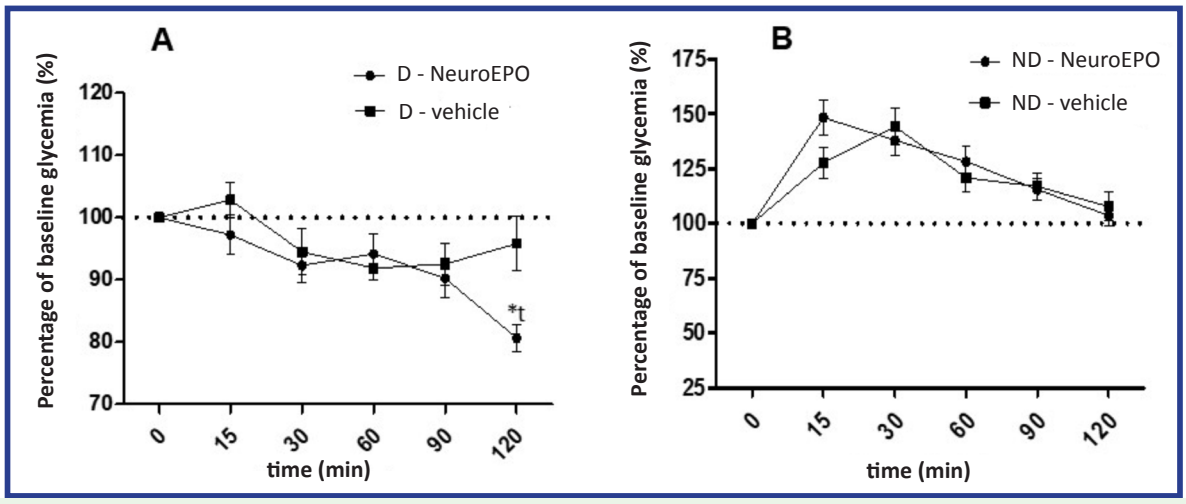


**Figure 2-** Glycemia of diabetic and non-diabetic rats injected with NeuroEPO or vehicle

Time 0 and dotted line correspond to the initial glycemia determined A) before and B) 30 minutes after subcutaneous injection of NeuroEPO (0.5 mg/kg) or vehicle. At each time, the average percentage concerning the initial glycemia and the standard error of the mean are shown.

A) Diabetic rats injected with NeuroEPO (D-NeuroEPO) or vehicle (D-vehicle). n=12 in each group. * $p< 0.05$ compared to initial glycemia (one-way ANOVA and Bonferroni test). t $p< 0.01$ compared to the vehicle group (two-way ANOVA and Bonferroni test).

B) Non-diabetic rats injected with NeuroEPO (ND-NeuroEPO) or vehicle (ND-vehicle) and a glucose tolerance test (2 g/kg of glucose orally). n=11 in each group. No significant differences were found between the groups (two-way ANOVA $p> 0.05$).

The results of experiment 3, in which the NeuroEPO or vehicle was administered to diabetic rats and then an insulin tolerance test was performed, showed that the glycemia of the group receiving NeuroEPO and insulin decreased significantly when compared to the group receiving vehicle and insulin (**Figure 3**).

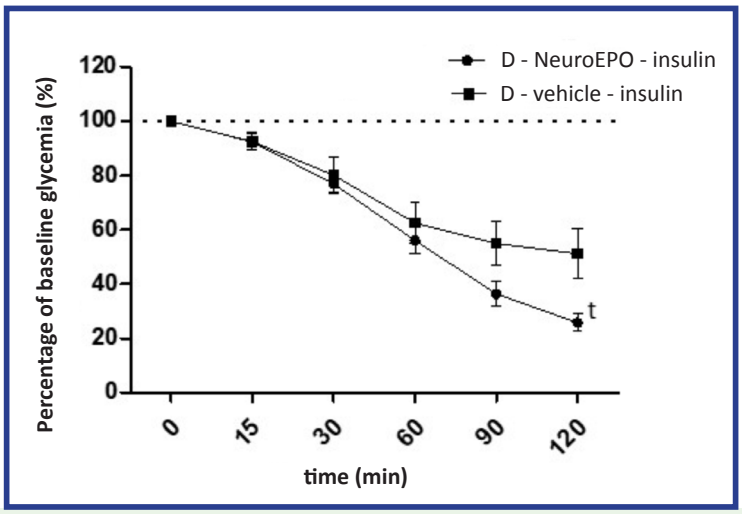


**Figure 3-** Glycemia of diabetic rats that received insulin after an injection of NeuroEPO or vehicle

Subcutaneous injection of NeuroEPO (0.5 mg/kg) or vehicle was applied. At 30 min, glycemia (initial glycemia, represented by time 0 and the dotted line) was determined and 0.75 IU/kg of insulin was administered subcutaneously. At each time, the average percentage concerning the initial glycemia and the standard error of the mean (n=11) are shown.

D-NeuroEPO-insulin: Diabetic rats injected with NeuroEPO and insulin. D-vehicle-insulin: diabetic rats injected with vehicle and insulin. t $p< 0.01$ compared to the vehicle group (two-way ANOVA and Bonferroni test).

## DISCUSSION

In the present work, the results showed that a subcutaneous injection of 0.5 mg/kg of NeuroEPO reduced the hyperglycemia of diabetic rats without insulin treatment but did not modify the glycemia of non-diabetic rats that received an oral glucose overload. In addition, a greater reduction in glycemia was observed in diabetic rats that received NeuroEPO and insulin, compared to those that received insulin.

Other studies have found a reduction in glycemia in diabetic rats after the administration of EPO, associated with a decrease in the expression of phosphoenolpyruvate carboxykinase (PEPCK) and the intensity of hepatic gluconeogenesis. An increase in the phosphorylated form of AMPK (AMP-dependent kinase), which is the most active form of the enzyme, has also been observed, associated with reduced PEPCK expression.[16,29]

Evidence indicates that decreased hepatic gluconeogenesis is one of the mechanisms by which EPO may have a hypoglycemic effect in diabetic rats. However, these investigations have been carried out after several applications of EPO, unlike the current study in which a single administration of a dose of NeuroEPO was performed.

One mechanism that could explain the reduction of hyperglycemia in diabetic rats after a single application of a dose of NeuroEPO is the stimulation of glucose consumption by tissues, not mediated by the action of endogenous insulin. It has been shown that the administration of streptozotocin to rats destroys the beta cells of the pancreas and generates a model of type 1 diabetes, in which the remaining levels of insulin in the blood can be only 4-8 % of those found in non-diabetic rats. [16,17] In addition, in non-diabetic rats, it has been observed that insulinemia does not increase after a single application of a dose of EPO.[16]

In a previous study, an increase in the expression of EPOR was observed in skeletal muscle of diabetic rats, which was reversed with insulin treatment as well as in muscle cells grown under conditions of high glucose concentration. [17] In addition, the expression of EPOR is known to be stimulated by pro-inflammatory cytokines in metabolic stress environments.[5] The above suggests that in diabetes there is an increase in the expression of EPOR, because of hyperglycemia and pro-inflammatory status, which may favor the action of exogenous EPO; a similar mechanism could explain the reduction of hyperglycemia of diabetic rats that received NeuroEPO, in which TPR may participate.

Results like those obtained in this research were published by Niu H. *et al,*[16] who observed a reduction in glycemia in diabetic rats after a single application of a dose of EPO. The researchers found an increase in the phosphorylated form of AMPK, associated with an increase in the expression of glucose transporters GLUT4 in the skeletal muscle of rats receiving EPO.

The interaction of EPO with TPRs activates multiple signaling pathways, in which the initial step is the autophosphorylation of Janus Kinase 2 (Jak2). As a result, three main pathways are activated, one of which is phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) or Akt. The PI3K/Akt pathway is related to inhibition of apoptosis, reduction of inflammation, increased blood flow, and recovery of damaged tissue.[5] In addition, PI3K/Akt is one of the pathways by which insulin increases the entry of glucose into muscle cells and adipocytes, by stimulating the translocation of GLUT4 to the membrane of these cells.[30,31] This shows that EPO may increase the entry of glucose into tissues by activating a signaling pathway common to that activated by insulin.

On the other hand, previous studies have shown that EPO does not reduce glycemia in non-diabetic rats,[13,14] similar to that observed in the present research with the use of NeuroEPO. The overall results suggest that hyperglycemia could cause overexpression of neuroEPO receptors, which would increase the NeuroEPO-receptor interaction and, consequently, the translocation of GLUT4 and the entry of glucose into tissues.

The fact that NeuroEPO did not modify glycemia in non-diabetic rats would provide security to non-diabetic patients receiving this substance for other causes since they would not suffer episodes of hypoglycemia as an adverse effect. However, this result should be deepened, since the current research only evaluates the influence of a single application of NeuroEPO on glucose tolerance of non-diabetic rats.

Another result of this research shows a greater reduction in glycemia of diabetic rats that received NeuroEPO and insulin, compared to those that only received insulin. Other studies have shown that the administration of EPO increases insulin sensitivity in diabetes, both in rats[14,16,29] and in humans.[31] In one such study, the administration of EPO to rats with type 2 diabetes increased the expression of EPOR in skeletal muscle, the phosphorylation, and activation of signal molecules, such as PI3K and Akt, the translocation of GLUT4 and autophagy, as well as reducing apoptosis.[29] The results indicate that EPO may reduce insulin resistance in diabetes by mechanisms involving the EPO-EPOR interaction.

In research in which an increase in insulin sensitivity associated with the administration of EPO has been found, the effect has been evidenced after repeated applications of this substance. However, the fact that a single application of NeuroEPO is sufficient to reduce hyperglycemia in rats with streptozotocin-induced diabetes may indicate a synergistic effect between NeuroEPO and insulin when administered in combination; under conditions of hyperglycemia, each is capable of triggering mechanisms that increase tissue glucose consumption.

The study focused on the influence of NeuroEPO on blood glucose levels and the results suggest an insulin-hydropic action in diabetic rats, but it is necessary to delve into this and other mechanisms of hyperglycemia reduction. In nondiabetic rats, the research was *limited* to evaluating the influence of a single application of a dose of NeuroEPO on glucose tolerance, but evaluations are required under normoglycemia conditions, especially with several applications of this substance and other dose levels.

## CONCLUSIONS

NeuroEPO has a hypoglycemic effect in diabetic rats due to an insulinotropic mechanism that shows synergism with insulin in the treatment of hyperglycemia. However, NeuroEPO does not influence the glucose tolerance in non-diabetic rats, at least immediately.

## RECOMMENDATIONS

We recommend to continue conducting research in the hypoglycemic effect of NeuroEPO, as it could be considered in future studies as an additional therapeutic strategy to insulin to improve glycemic control and reduce complications in diabetic patients.

## ACKNOWLEDGEMENT

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Authorship contribution**

Tammy Fernández Romero: Conceptualization, data curation, formal analysis, acquisition of funds, research, methodology, project management, writing–original draft, writing–review and editing.

Sonia Clapés Hernández: Conceptualization, data curation, formal analysis, acquisition of funds, project management, writing–original draft, writing–review and editing.

Carlos Luis Pérez Hernández: Data collection, formal analysis, writing–original draft, writing–review and editing.

José Javier Barreto López: Data consolidation, formal analysis, writing–original draft, writing–review and editing.

Gisselle Fernández Peña: Data collection, formal analysis, writing–original draft, writing–review and editing.

All authors have participated in the discussion of the results and have read, reviewed, and approved the final text.