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Research Article

Effect of Metformin on the Expression of α-7nAch Receptors in Alloxan-induced Diabetic Mouse Model: A Preliminary Study

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Abstract

Background: Biological evidence confirmed the link between diabetic disease and cognitive dysfunction, but the exact mechanism is not fully understood. Alpha-7 nicotinic acetylcholine receptors (α7-nAChRs) are transmembrane receptors activated in response to neurotransmitters and play an important role in the mammal's cognitive function. ***Objectives***: To study the effect of metformin on α7-nAchRs expression in brain and spleen tissues of diabetic mice. ***Methods***: Forty mice were allocated into 4 groups and were subjected to 7- and 12-week interventions. G1 was healthy, G2 experimentally induced diabetes without treatment, and G3 and G4 were diabetic mice treated with metformin (50 and 100 mg/kg, respectively). Blood glucose level was monitored during treatment. α7-nAChRs expression was evaluated by the immunohistochemical method after ending treatment. ***Results***: In brain tissue, G1 showed strong expression (+2.5), and G2 weak (+1) for both periods. G3 and G4 revealed moderate (+1.5) staining in 7 weeks and weak (+1) in 12 weeks. In spleen tissues, G1, G2, and G3 showed strong staining (+3), but G4 revealed moderate (+1.5) in 7 weeks and moderate to strong (+3/+2.5) in 12 weeks. ***Conclusions***: Diabetic mice exhibit low expression of α7nAChRs in the brain tissue, but those receptors were moderately recovered at 7 weeks of therapy duration by metformin, which may be a good therapeutic option for the management of dementia.

Keywords: α7-nAChRs, Dementia, Diabetic mouse model, Metformin.

تأثير الميتفورمين على التعبير عن مستقبلات α-7nAch في نموذج الفأر السكري الناجم عن الألوكسان: دراسة أولية

الخلاصة

الخلفية: أكدت الأدلة البيولوجية العلاقة بين مرض السكري والخلل الوظيفي الإدراكي، لكن الآلية الدقيقة ليست مفهومة تماما. مستقبلات أستيل كولين النيكوتين ألفا 7 (α7-nAChRs) هي مستقبلات عبر الغشاء يتم تنشيطها استجابة للناقلات العصبية وتلعب دورا مهما في الوظيفة المعرفية للثدييات. **الأهداف**: دراسة تأثير الميتفورمين على تعبير α7-nAchRs في أنسجة الدماغ والطحال للفئران السكرية.**الطرائق**: تم تقسيم أربعين فأرا إلى 4 مجموعات ,خضعوا للتجربة لمدة 7 و 12 أسبوعا. كان G1 فئران اصحاء و-G2 فئران استحث فيها مرض السكري باستخدام الالوكسان ولم تخضع للعلاج بالميتفورمين، و G3 و G4 كانتا من الفئران المصابة بالسكري عولجت بالميتفورمين (50 و 100 مجم/كجم، على التوالي). تمت مراقبة مستوى الجلوكوز في الدم أثناء العلاج. تم تقييم تعبير α7-nAChRs بالطريقة الكيميائية المناعية النسيجية بعد انتهاء مدة التجربة. **النتائج**: في أنسجة المخ، أظهر G1 تعبيرا قويا (+2.5)، و G2 ضعيفا (+1) لكلتا الفترتين. كشف G3 و G4 عن تلطيخ معتدل (+1.5) في 7 أسابيع وضعيف (+1) في 12 أسبوعا. في أنسجة الطحال، أظهر G1 و G2 و G3 تلطيخا قويا (+3) ، لكن G4 كشف عن معتدل (+1.5) في 7 أسابيع ومعتدل إلى قوي (+3 / + 2.5) في 12 أسبوعا. **الاستنتاجات**: تظهر الفئران المصابة بالسكري تعبيرا منخفضا عن α7nAChRs في أنسجة المخ، ولكن تم استرداد هذه المستقبلات بشكل معتدل في 7 أسابيع من مدة العلاج بالميتفورمين، والذي قد يكون خيارا علاجيا جيدا للخرف.

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INTRODUCTION

Cognitive dysfunction is well-established as a consequence of diabetes [1,2]. Diabetes is nearly doubling the risk for dementia [3,4], particularly in older type 2 diabetes. Despite the link between diabetes and dementia being well known, there is significant disagreement about whether this relationship also extends to cognitive defect [3]. This is because the exact biological mechanisms underlying this relation and the role of glycemic control and diabetic complications in dementia progression are less well understood [5]. Several literatures have demonstrated that diabetes and its microvascular complications, the treatment by insulin and other anti-diabetic drugs, can induce mild to moderate neurocognitive dysfunction that is originally a result of structural and functional modifications in the central nervous system (CNS) leading to neurological diseases such as dementia [6]. Dementia, commonly including Alzheimer's disease (AD), can affect memory and other cognitive function domains

[7], interfering with a person's ability to perform routine daily activities [7–9]. The latest surveys of published papers and epidemiological data display the increasing risk of developing AD in people with type 2 diabetes [1,10]. For instance, the study of Biessels and colleagues reported that diabetes is associated with long-term complications in the brain, manifested by impairment of cognitive ability and more abnormalities seen with brain imaging compared to people without diabetes [11]. The study of Kopf *et al*. also reported that the risk of AD was observed in diabetic populations [12]. Nicotinic acetylcholine receptors (nAChRs) are channels that serve as ligand-gated cations, which are highly distributed within the body and nervous system, and the most abundant types are α4β2 and α7β2 subtypes in the brain [13]. These receptors play a vital role in the learning and memory processes, adjusting the neurotransmission and synaptic elasticity pathways [14]. α-7subtype is highly expressed in the basal forebrain cholinergic neurons that project to the hippocampus and cortex of normal and AD brains [14,15]. It is implicated in the cognitive functions of the central nervous system. Modulation of α7nAChR is considered to be a perspective for the treatment of cognitive disorders such as AD or schizophrenia. Agonists of α7nAChRs can penetrate through the blood-brain barrier. They are objects of pharmacologists' interest as they are suitable for cognitive function amelioration in patients suffering from AD and schizophrenia [16]. Metformin is an orally administered drug used for lowering blood glucose concentrations in patients with type 2 diabetes mellitus (T2DM), particularly in those overweight and obese as well as those with normal renal functions. Pharmacologically, metformin belongs to the biguanide class of anti-diabetes drugs. It has been known that the anti-hyperglycemic effect of metformin is mainly due to the inhibition of hepatic glucose output, and therefore, the liver is presumably the primary site of metformin function [17]. Some studies give evidence that metformin might have beneficial effects on cognitive impairment and memory loss [10]. Zhao and colleagues evaluated the ameliorative effects of metformin on seizures, cognitive impairment, and brain oxidative stress markers observed in pentylenetetrazole-induced kindling animals. The authors confirmed that metformin suppressed the progression of kindling, ameliorated the cognitive impairment, and decreased brain oxidative stress. These results led to the conclusion that metformin may be a potential preventive agent against cognitive impairment [18]. Recently, several papers have examined the use of metformin in the treatment of neurodegenerative diseases such as AD, amnestic mild cognitive impairment, and Parkinson's disease [19]. A large body of data on metformin use in humans and animals with neurodegenerative diseases exists, but metformin's therapeutic use is not yet accepted since the results are often conflicting [20]. The current study aimed to investigate the effect of metformin on the expression of α7nAChR in brain and spleen tissue of mice-induced diabetic disease.

METHODS

Materials

Alloxan monohydrate (2,4,5,6-tetraoxyprimidine 5,6-dioxyuracil) was obtained from Alpha Chemica, India; metformin hydrochloride (1,1-dimethylbiguanide hydrochloride) from Sigma-Aldrich, Germany; formaldehyde solution 10% (Alpha Chemica, India), ethanol (Sigma-Aldrich, Germany), xylene (Alpha Chemica, India), and paraffin wax (Alpha Chemica, India); the kit of immunohistochemical (IHC) assay was obtained from Genemed Biotechnology, INC, USA; and anti-α7nAChR from MyBiosource, USA.

Study design

Forty albino male mice were subjected to experiment for 7 and 12 weeks; their ages were 2-4 months, and their body weights ranged from 20 to 30 gm. Mice were divided into 4 groups, with 10 in each cage. Group G1 was healthy without diabetes, and the other 30 were subjected to the induction of diabetes. Diabetic mice were distributed into 3 groups. The group of mice (G2) was diabetic mice but never treated with metformin. Groups G3 and G4 included mice treated with metformin in a dose of 50 and 100 mg/kg once daily, respectively, for 7 weeks as a first program. After finishing the first program, two mice were euthanized, and the obtained tissues were preserved in paraffin wax for the P-embedded staining assay and immunohistochemical analysis (IHC) of α7nAChR in the brain and spleen. The remaining mice continued to receive treatment and completed 12 weeks from the first day and were termed a "second program" in a dose administered on alternate days to observe the effect of hyperglycemia and the impact of metformin on receptor expression simultaneously. After finishing the second program, two mice from each group were euthanized, and the obtained tissues were preserved until analysis.

Induction of diabetes

Alloxan monohydrate solution in a dose of 150 mg/kg was prepared in normal saline solvent and injected subcutaneously into mice as described by a study of Akinola *et al*. [21]. The blood glucose level was checked daily in the morning after fasting using the ACCU-CHEK Active glucose test device. After 14 days, the dose of 75 mg/kg alloxan was injected. After 21 days, hyperglycemia (glucose > 200 mg/dl) was monitored in the mice treated.

Measurement of blood glucose

In all groups, fasting blood sugar (FBS) was analyzed before initiating the experiment program, and after induction of diabetes and during the drug treatment. The presence of diabetes mellitus was confirmed by the high blood glucose levels. Also, FBS was monitored once a week for all mice during the duration of the two programs.

Preparation and staining of tissue sections

Tissues were dipped in 10% formaldehyde solution for preserving their contents from the environmental effects and embedded in paraffin wax for long-term maintained specimens. The sample slide was prepared by cutting 4 µm thick sections of tissue and incubating them at 100°C for 30 minutes to retrieve the antigenic epitopes of the receptor protein. The indirect IHC staining protocol was applied according to their manufacturer's reference**.** The reading of the results had been done in two manners: qualitative (positive or negative) scored results and quantitative (degree of the positive findings +1, +2, +3), which was called the combinative semi-quantitative scoring under the histopathologist physician [22].

Ethical considerations

This study was approved by the Institutional Review Board of Ibn Sina University of Medical and Pharmaceutical Sciences, Baghdad, Iraq (ISU.6.2.24), on 22/9/2024, in accordance with the Helsinki Declaration, 2004.

RESULTS

At the first program, as seen in Table 1, the brain tissue receptors showed strong staining in healthy groups and weak staining in diabetic mice for both 7 and 12 weeks of duration, as seen in Figure 1 (a and b) and Figure 2 (a and b), respectively.

Table 1: Score of Immunohistochemistry staining for α-7nAchRs in brain tissue

Brain	Intensity Semi-Quantitative Scores			
	P1 (7 weeks)	Average of staining score and diagnosis	P2 (12 weeks)	Average staining score and diagnosis
G 1	+++ ++	2.5/S	+++ ++	2.5/S
G 2	+/± +/±	1/W	± ±	< 1/VW
G 3 (50 mg)	+ +++	1.5/M	+ +	1/W
G4 (100 mg)	+ ++	1.5/M	+ +	1/W

The result represents duplicate reading. P1: program 1 duration, P2: program 2 duration, S: strong, M: moderate, W: weak, VW: very weak scores.

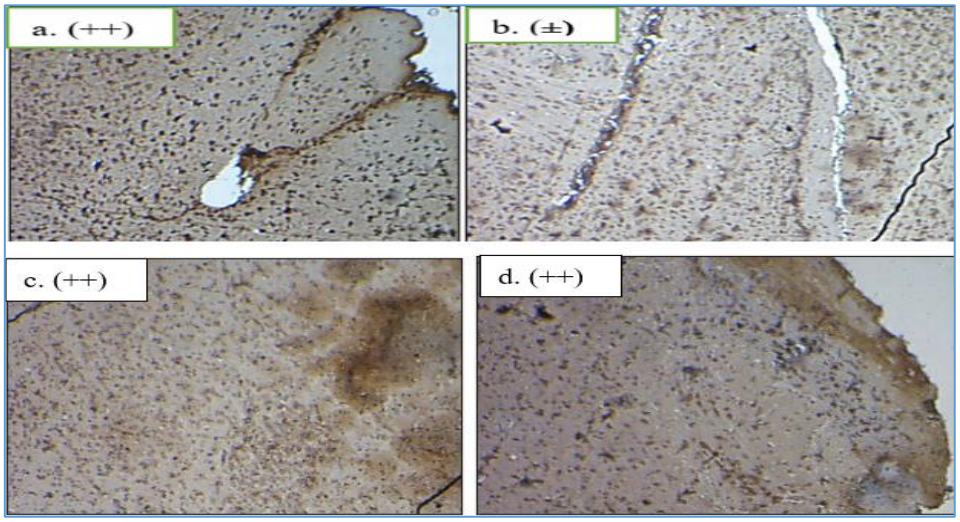

Figure 1: Microscopic images (10x) of tissue brain of mice at 7 weeks duration. **a**) strong staining of α-7nAChRs in G1 healthy mice. **b**) weak staining in injured mice G2. **c**) and **d**) moderate staining of the receptor at 50 and 100 mg/kg dose of metformin. The brown color represents the α-7nAChRs antigen–antibody binding complex.

A dose of 50 mg/kg G3 showed a moderate rate of staining at the 7-week treatment duration, as seen in Figure 1c, and weak staining at 12 weeks, as seen in Figure 2c. Similarly, the dose of 100 mg/kg of G4 also showed moderate staining at 7 weeks of therapy, as shown in Figure 1d, and weak staining at 12 weeks duration, as seen in Figure 2d. In spleen tissues, as shown in Table 2, the intensity of IHC staining for α-7nAchR receptors appeared as strong staining in healthy mice for 7- and 12-week durations, as seen in Figure 3a and Figure 4a, respectively. Also, the diabetic mice in G2 showed strong staining for both duration treatment groups, as seen in Figures 3b and 4b. The staining of 50 mg/kg therapy G3 showed strong staining for the two durations, as seen in Figures 3c and 4c, respectively. However, with the 100 mg/kg treatment G4, the staining of receptors was at a moderate rate at 7 weeks and moderate to strong at 12 weeks, as seen in Figures 3d and 4d, respectively.

DISCUSSION

Typically, the abundance of α-7nAchR receptors is vastly present and distributed in mammals' brains; it shows a highly to moderate distribution in the hippocampus [23–25]. Our result agreed with this scientific fact, where healthy mice revealed a strong staining. However, diabetic mice showed weak staining at the two durations of metformin treatment.

Several earlier studies reported that the decline in nicotinic receptors, mostly the α-7nAchR, is linked with aging [24] and also reduced particularly in brain AD patients.

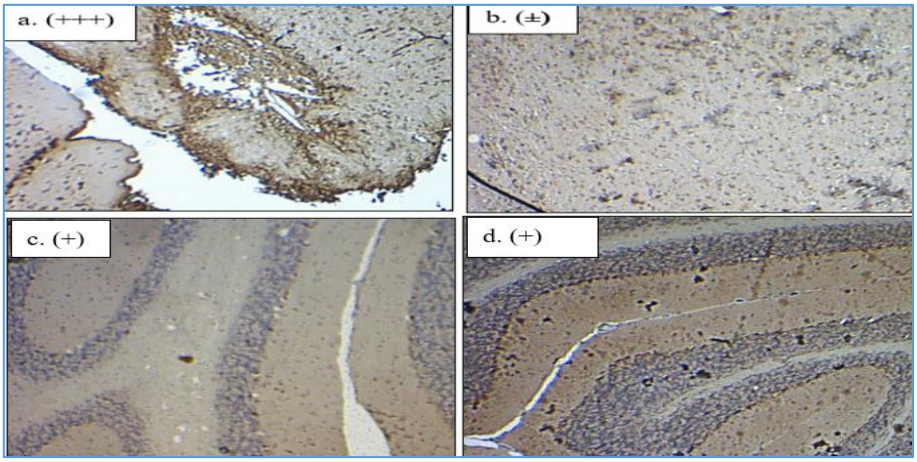

Figure 2: Microscopic images (10x) of tissue brain of mice at 12 weeks duration. **a**; strong staining of α-7nAChRs in G1 healthy mice. **b**; very weak staining in injured mice G2. **c.** and **d**. weak staining of receptor at 50 and 100 mg/kg dose of metformin. The brown color represents the α-7nAChRs antigen–antibody binding complex.

This deficit appears at the early stage of disease and relates to progressive loss of cognitive functions [24–26]. Thus, the activation of these receptors is a beneficial therapeutic strategy for cognitive dysfunction [24–27]. The less availability of receptors in brain tissues of induced-diabetic disease mice in this work confirms the fact that diabetic disease is greatly led to one of its complications, which is a cognitive decline. While the moderate staining at 7 weeks for 50 and 100 mg/kg treatment doses confirmed the recovery of the abundance of receptors in brain tissues for both treatment groups to levels less than in normal healthy mice, it indicates that metformin may be a good therapeutic agent for the α7nAchRs abundance and prevent the progress of cognitive dysfunction complications. The weak staining of G3 and G4 at 12 weeks refers to the reduced dose treatment of three times a week.

Table 2: Scores of Immunohistochemistry staining for α-7nAchR in spleen tissue for all studied mice

Spleen tissues	Intensity Semi-Quantitative Scores			
	P1 (7 weeks)	Average of staining score and diagnosis	P2 (12 weeks)	Average of staining score and diagnosis
G1	+++	3 / S	+++	3 / S
	+++		+++	
G2	+++	3 / S	+++	3 / S
	+++		+++	
G3 (50 mg)	++	2.5 / S	+++	3 / S
	+++		+++	
G4 (100 mg)	+	1.5 / M	++	2.5 / S
	++		+++	

The result represents duplicate reading. P1; program 1 duration, P2; program 2 duration, S; strong, M; moderate.

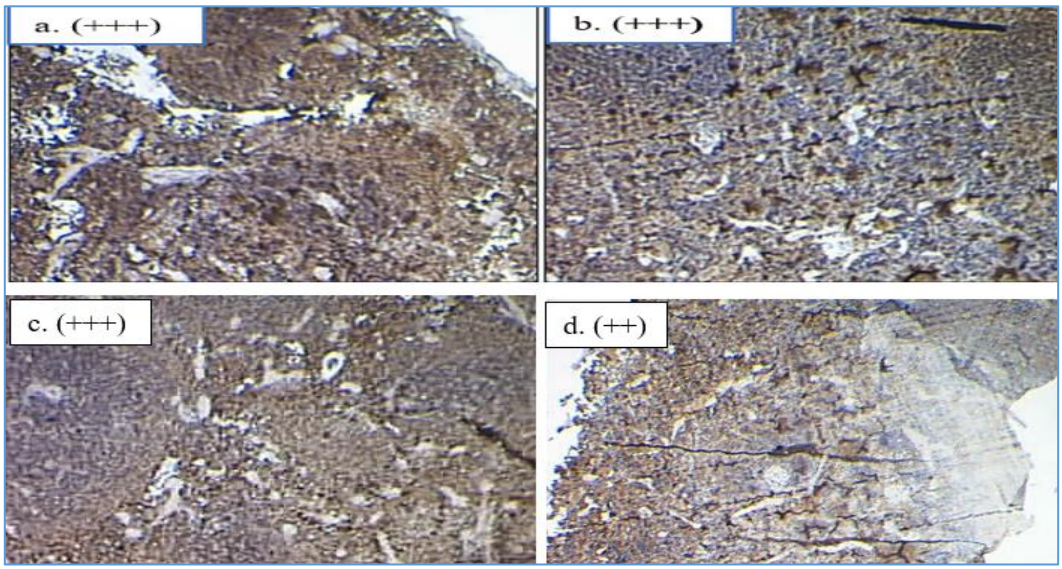

Figure 3: Microscopic images (10x) of spleen mice tissue at 7 weeks duration. **a, b, and c**; strong staining of α-7nAChRs in G1 healthy mice, injured mice G2 and 50 mg/kg dose treatment respectively. **d:** moderate staining of receptor in 100 mg/kg dose of metformin. The brown color represents the α-7nAChRs antigen–antibody binding complex.

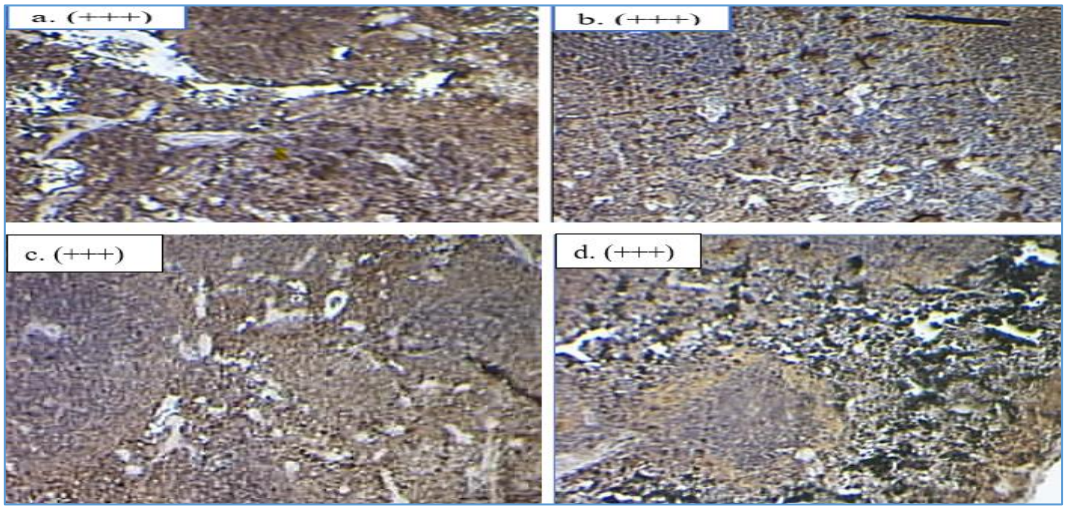

Figure 4: Microscopic images (10x) of spleen mice tissue at 12 weeks duration. **a, b, c and d**; strong staining of α-7nAChRs in G1 healthy mice, injured mice G2, 50, and 100 mg/kg dose treatment groups respectively. The brown color represents the α-7nAChRs antigen–antibody binding complex.

These results indicate that the detrimental effect of hyperglycemia on the abundance of α7nAchRs has been protruded when the dose of metformin is reduced or altered to be received only three times a week. The spleen organ is considered the major source for many inflammatory factors that participated in the directness of the most systemic inflammation [28]. The receptors of α-7nAchRs in spleen tissues are highly expressed due to their critical anti-inflammatory role. The acetylcholine (Ach) is the main neurotransmitter secreted by the vagal nerve [29–31], which is the longest and most distinct nerve in the parasympathetic system that innervates most of the peripheral organs, including the spleen [28]. It is believed that the neurotransmitter Ach controls the functions of the immune cell by their binding to α7nAChRs at the splenic myeloid cell (macrophage) to regulate splenic nerve function, resulting considerably in the attenuation of the release of many of the pro-inflammatory cytokines, leading to reduced inflammation [29,31-33]. Therefore, we found the strong staining level in control healthy mice, which represents the high expression of α-7nAChRs, as most previous studies reported. However, the staining was also strong in diabetic mice. This means that the diabetic disease had no effect on the expression of α7nAChRs in spleen tissue. Furthermore, the treatment dose of 50 mg/kg of metformin also showed no reducing or increasing effect on the receptor abundance for both 7- and 12-week treatment durations, but a dose of 100 mg/kg treatment reduced the availability of receptors to a moderate rate in average staining (+1.5) at 7 weeks, and after 12 weeks, it appeared in less intensity between moderate and strong in average (+2.5) stain, as seen in Table 2. This indicates that the metformin drug had no effect on a low dose, but at the high 100 mg dose of treatment, it exhibits a relatively negative effect, which reduces the availability of receptors in spleen tissues. Our study discovered a contradictory behavior of metformin on the abundance of the α7nAChRs receptor. In the brain it had a beneficial effect, but in the spleen, it had no effect in low doses, but it had a relatively hazardous side effect in a dose higher than 100 mg/kg. Such discrepancies should be taken into consideration. We did not find a literature review talking about how the metformin could influence the α7nAChRs receptors, and the exact mechanism effect of metformin is still poorly understood. Instead, we found a study of Hashish, aimed to investigate the possible pathological effect of metformin on the spleen in normoglycemic rats and its possible effect on a family of apoptosis regulators expression called B-cell leukemia/lymphoma-2 (Bcl-2). The rats treated with metformin showed distorted splenic contour. The white pulp appeared irregular; some cells were degenerated, with vacuolated cytoplasm and pyknotic nuclei. The study concluded that metformin should be used with caution to avoid its hazardous effect, especially in the spleen tissue [34]. Although the aim of the Hashish study differs from our objective, we agreed on this investigation about the negative effect of metformin on the splenic tissues of mice.

Study limitations

This study lacks statistical analysis since the experiment had no adequate data, so it was entitled as an experimental preliminary study. More investigations are needed, firstly to study the receptors at the diabetic disease state alone. Secondly, for studying the impact of this medication on the receptor availability at disease condition with metformin receiving.

Conclusion

The study concludes that the reduction of α7nAChRs expression in brain tissues indicates that diabetic disease could lead to cognitive dysfunction. Different mechanisms of metformin were observed in the brain; it recovers the receptors in different degrees depending on the dose of metformin, which stimulates the genetic expression of α7nAChRs, and it could be considered a good therapeutic agent for cognitive dysfunctions. While in spleen tissue, metformin reduces the expression of receptors at high doses without the influential role of hyperglycemia.

Conflict of interests

The authors declared no conflict of interest.

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The authors did not receive any source of funds.

Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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