Impact of Metformin on Cognition Impairment in Type 2 Diabetic Male Albino Rats

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Abstract

Background: There is controversy around metformin benefits, an insulin-sensitizing drug that is frequently administered, for enhancing cognitive performance in type 2 diabetic mellitus (T2DM) patients which increases cognitive impairment risk.

Objectives: This study aims to assess the ameliorative effects of metformin on T2DM-induced cognitive impairment.

Materials and methods: Rats were randomly assigned to three groups: group I (Control), group II (Diabetic), and group III (Diabetic-Metformin). Streptozotocin (STZ) (30 mg/kg, i.p.) administered as single dose to groups II and III following ten-week high-fat diet. Group III received metformin for ten weeks. Hippocampal memory examined using T Maze and novel object cognition tests were performed before animal scarification. Hippocampal samples were extracted at experiment end for biochemical (TNFα, IL-1β levels), histological and immunohistochemical (Bcl2/Bax ratio) studies.

Results: Group III showed a significant increase in spontaneous alternation in T-maze test (76.67% ± 15.02) compared to group II (6.67% ± 10.32) ($p < 0.001$). Significant increase in discrimination ratio in novel object recognition test was observed in group III after treatment (0.111 ± 0.02) compared to before treatment (-0.0466 ± 0.015) ($P < 0.05$). There was increase in Bcl-2/Bax ratio expression (2.794 ± 0.59) after treatment compared to before treatment (0.294 ± 0.08) ($P < 0.001$). Metformin decreased levels of TNFα and IL-1β in diabetic rats from (753.2 ± 86.3), (455.7 ± 43.6) respectively, to (372.1 ± 48.1), (220.1 ± 16.3) ($P < 0.001$).

Conclusion: Our results suggest that metformin may be promising drug for improving T2DM-induced cognitive dysfunction by reducing harmful pathophysiological effects.

Keywords: T2DM; Apoptosis; Inflammation; Metformin; Cognition impairment; BCL2/BAX ratio.

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Introduction

Diabetes mellitus (DM) is a common metabolic illness marked by abnormalities in the metabolism of proteins, lipids, and carbohydrates as well as consistently high blood sugar levels **(Styne, 2023)**. Regarding the percentage of people with diabetes worldwide, Egypt is ranked in the top 10 by the International Diabetes Federation (IDF) 2.2 million Egyptians have prediabetes and 7.5 million have diabetes, according to a 2013 IDF report. Additionally, reports suggest that 43% of Egyptians with diabetes and the majority of those with prediabetes may not have received a correct diagnosis **(Styne, 2023)**.

Three pathophysiologic abnormalities are identified in T2DM: inadequate insulin secretion, peripheral insulin resistance, and elevated glucose synthesis in the liver **(Galicia-Garcia et al., 2020)**. Insulin resistance leads to decreased peripheral glucose uptake and impaired inhibition of endogenous glucose production, both at rest and following meals **(Himanshu et al., 2020)**. Poor DM control over a long period can cause severe complications such as blindness, neuropathy, cognitive impairment, heart disease, kidney failure, sexual dysfunction, severe infections, and skin disease **(Farmaki et al., 2020)**. Several studies concluded that DM has a hazardous effect on hippocampal region of the brain thus increase risk of cognition impairment specially on the aging diseased group of population. To effectively manage this potentially distressing consequence of T2DM, it is essential to understand the manifestation of cognitive impairment, its pathogenesis, and the factors associated with this issue **(Damanik and Yunir, 2021).**

Metformin Synthetic dimethyl biguanide has been used in clinical practice for more than 60 years. Approximately 125 million people use metformin worldwide. This could be attributed to the possibility of a small weight reduction and its minimal risk of hypoglycemia, the low cost, acceptable tolerability, long-term cardiovascular and general safety so metformin is regarded as the drug of choice and first line of treatment for the condition **(LaMoia and Shulman, 2021)**.

There is controversy on the impact of metformin on cognitive impairment. Previous studies were on alignment to the benefits of metformin in reducing the risk dementia, but not Alzheimer's disease **(J. H. Zhang et al., 2022)**. This study aimed to consider metformin as a promising drug for improving DM-induced cognitive dysfunction by reducing the harmful pathophysiological effects induced by DM on the hippocampus.

Materials and Methods

In the Pharmacology Department of the Faculty of Medicine at South Valley University, we conducted an experimental randomized control trial from November 2022 to May 2023. The South Valley University Faculty of Medicine's Animal Ethical Committee approved all experimental procedure. **Ethical approval code:** SVU-MED-PHA006-2-22-1-317.

1. Experimental animal

The study included eighteen Male albino Sprague Dawley rats, two months old and weighing 160±10 g. Rats were obtained from the Egyptian Company for the Production of Antisera, Vaccines, and Drugs Helwan, Egypt. They were housed in polypropylene cages with stainless steel covers and three per cage (41 × 34 × 16 cm), in a standard environment in a room with a temperature at 22±2 °C and 12/12 h day/night cycle, animals were given free access to water and a standard pellet diet (73% carbohydrate, 21% protein, 3% fat, 3%fibers, vitamin, and minerals). All rats were adapted for a one-week duration prior to the start of the experiment.

2. Experimental design

Randomly, rats were divided into three groups, six rats in each:

Group I (Control): Rats of this group were fed on normal palatable diet (NPD) and received normal saline all over the experiment.

Group II (Diabetic): Rats of this group were given a high-fat diet (20% fructose, 5% sucrose, 15% casein, 5% fiber, 20% coconut oil, 3.5% soybean oil, 22.5% starch, 5% wheat, 1.2% vitamin mix, 2.4% mineral mix and 0.4% salt) for ten weeks then fasted overnight and received an injection of freshly prepared single dose of streptozotocin I.P. (30 mg/kg diluted in a volume of 1 ml M citrate buffer in 4.5 PH). Rats of this group continued on a high-fat diet till the end of experiment **(Zeinab et al., 2020).**

Group III (Diabetic-Metformin): Animals of this group underwent induction of T2DM as Group II and they simultaneously received metformin (300 mg/kg/day, orally) dissolved in saline from the 12th week of experiment and given once daily and repeated till the end of the experiment **(Mostafa et al.,2016).**

3. Assessment methods:

3.1. Body weight assessment: Each rat's body weight in all groups was measured weekly by a digital scale. and recorded.

3.2. Blood glucose measurement: Drops of blood were obtained from rat`s tail vein for monitoring blood glucose weekly.

3.3. Behavioral assessment: Learning and memory were evaluated prior to animal scarification using the T Maze spontaneous alternation test and the novel object recognition test.

3.3.1. T Maze Spontaneous Alternation: Using a discrete trial, the T-Maze concept was carried out in accordance with the guidelines of a prior research by **(Grzeda et al., 2007).**

3.3.2. Novel object recognition test: The intermediate-term memory can be assessed on a novel object recognition test, the methodology of a prior study by **Hoffman and Basurto (2014)** was followed when performing the novel object recognition test.

4. Sampling

After the experiment was over, the animals got unconscious with ether and had intracardiac perfusions of 10% neutral buffered formalin and saline. The brain was retrieved, and the hippocampus was removed bilaterally. Tissue specimens were immersed in liquid nitrogen and kept at -80 °C in order to quantify TNF-α and IL1β, two indicators of inflammation. Several other tissues were placed in 10% paraformaldehyde for analysis using immunohistochemistry and histology.

4.1. Enzyme-Linked Immunosorbent Assay (ELISA) for measuring the level of TNF-α and IL1β : After dissecting the tissues into small pieces and rinsing them in ice-cold PBS (0.01M, pH=7.4), all excess blood has been totally removed. Using a glass homogenizer on ice, weigh the tissue pieces before homogenizing them in PBS (tissue weight (g): PBS volume (mL) = 1:9). To obtain the supernatant, the homogenates are centrifuged for 15 minutes at 50,000 ×g. the amounts of protein measured using the Bradford Protein Assay **(Bradford, 1976)** by Bradford Protein Colorimetric Assay Kit (Elabscience Biotechnology Inc., Catalog no: E-BC-K168-M, USA). This assay is based on the principle that Coomassie Brilliant Blue, a dye molecule, shifts its absorption spectrum when it binds to proteins. The color change from brown to blue occurs when the dye binds to protein, shifting its absorption maximum from 465 nm to 595 nm. Measurement of the blue-colored solution's absorbance at 595 nm and comparison with a standard curve prepared with known protein concentrations yield the amount of protein in a sample. TNF-α ELISA kit (Elabscience Biotechnology Inc., Catalog no: E-EL-R0019, USA) and IL-1β ELISA kit (Elabscience Biotechnology Inc., Catalog no: E-EL-R0012, USA) uses Sandwich-ELISA as the method. Rat TNF-α and IL1β-specific antibody has been pre-coated on the Micro ELISA plate (MR-96A, Sunshine, India) included in this kit. Wells designated for Micro ELISA plates are filled with standards or samples, then the corresponding antibody is added. Following the addition of an avidin-Horseradish Peroxidase (HRP) conjugate and abiotinylated detection antibodies specific for Rat TNF-α and IL1β,

well of each micro plate is incubated then free components are eliminated during incubation. Next, the Substrate Reagent is introduced into every well; the only wells that will become blue are those that have the Avidin-HRP conjugate, biotinylated detection antibody, and Rat TNF-α and IL1β. When the Stop Solution is added, the enzyme-substrate reaction will be stopped and turn yellow (**Chennaoui et al., 2015**).

5. Histopathological and Immunohistochemical Assessment:

5. 1. General histological examination: Bancroft and Stevens (2013) explained how to prepare tissues for histology. In brief a sample of the hippocampus was cut into slices that were 3–4 mm thick, fixed in 10% neutral buffered formalin (10% NBF), dehydrated in graded ethanol series, cleared in xylene, and embedded in paraffin. Hematoxylin and Eosin staining was applied to the paraffin blocks after they were divided into sections using a microtome at a thickness of 4-6 μm in order to examine the overall structure of the tissue. Sections stained with H&E were evaluated using a Leica microscope (CH9435 Heerbrugg, Leica Microsystems, Switzerland). **Malcok et al. (2021)** scored and documented the histopathological growth of the pericellular region, vacuolization, and pyknosis in the hippocampal tissues.

5.2. Immunohistochemistry Staining Protocol: The anti-apoptotic protein Bcl-2 and the apoptotic protein Bax were shown to be expressed in hippocampus tissue using immunohistochemistry (IHC). Rabbit anti-Bax Polyclonal (Cat. No. SAB2109199 from Sigma-Aldrich Corp., St. Louis, MO, USA) Dilution: 1:200 and mouse anti-Bcl2 monoclonal antibody (Cat. No. E-AB-22490 from Elabscience, USA) Dilution: 1:500 was used in according to manufacturer's instructions.

In order to inhibit endogenous peroxidase activity, 5-μm-thick hippocampal slices were deparaffinized, rehydrated, and subjected to a 3% H2O2 pretreatment. Microwaving slides in a 10 ml sodium citrate buffer for 10 minutes was the method used to retrieve the antigen. The slides were immersed in Tris-buffer saline, then incubated once more with the secondary antibody after the primary antibody had finished. Diaminobenzidine (DAB, Sigma) substrate chromogen solution was applied to the slides, and hematoxylin was used as a counterstain afterward. Leica microscopes at Cairo University's Faculty of Veterinary Medicine were used to capture photos of nine distinct fields at 400 times magnification.

Image-JR software was used to measure and quantify the region of positive immune-expression.

Statistical analysis

After verifying the information, the researcher created it. To do the statistical analysis, SPSS version 25* was used. Unique statistical data: This involved calculating the means and standard deviations (SD). The significance test was performed using paired samples T-test analysis for continuous variables with two categories, in order to compare the means of normally distributed data. The post-hoc test was calculated using Tukey corrections, a p-value of less than 0.05 was considered significant, and the ANOVA test was used to find the mean differences between the data while using continuous variables that had more than two categories.

Results

1. Body weight

Injections of STZ and a high-fat diet were administered to rats until week 11 in the diabetic and diabetic-metformin groups. With a statistical significance ($p < 0.05$), their mean body weights were considerably greater than the controls. Comparing the diabetic-metformin group and the diabetic rats to the control group, the average body weight of the latter group decreased non-significantly ($p < 0.05$) between weeks 11 and 17. The diabetic and diabetic-metformin groups differed significantly from one another ($p < 0.05$). However, by week 18, there was no statistically significant difference between the control, diabetic-metformin, and diabetic groups ($p > 0.05$) **(Table 1).**

2. Random blood sugar and HbA1c

The mean blood glucose levels of the rats in the diabetic and diabetic-metformin groups did not alter statistically significantly following HFD and STZ injections until week 11. ($p < 0.05$). Beginning in week 11, the mean blood glucose levels of the diabetic rats increased significantly ($p < 0.05$) in comparison to the control group. The injection of metformin to diabetic rats significantly reduced their raised mean blood glucose levels as compared to diabetic rats ($p < 0.05$), but there was no significant difference between the control and diabetic-metformin groups ($p > 0.05$) **(Table.1).** As indicated by **Table 1**, The HbA1c in the diabetic rats significantly increased to 5.55 ± 0.104% ($p = 0.001$) from 3.65 ± 0.137% in the control group. It is noteworthy that, as indicated in (**Table.1)**, the HbA1c in the diabetic-metformin group was significantly lower than that of the diabetic group (P=0.001).

Table 1. Comparison of body weight, blood glucose, and HbA1c results across different studied groups

Parameters	Group I: Control N=6	Group II: Diabetic N=6	Group III: Diabetic-metformin N=6	P value
Body weight (mg)				
At the start	**190±15.4**	**193±15**	**210±14.2**	**0.675**
P value**	**I vs. II=0.9**	**II vs. III=0.6**	**I vs. III =0.8**	
At the 11th week	**141.5±15.4**	**345±9.2**	**315±5.7**	**0.001***
P value**	**I vs. II=0.001**	**II vs. III=0.687**	**I vs. III =0.001**	
At the 20th week	**185.6±18.9**	**343.5±11.14**	**201.8±3.76**	**0.001***
P value**	**I vs. II=0.001**	**II vs. III=0.001**	**I vs. III =0.553**	
Blood glucose level (mg/dl)				
At the start	**97.6±19**	**97±16**	**96±18**	**0.876**
P value**	**I vs. II=0.964**	**II vs. III=0.887**	**I vs. III =0.753**	
At the 11th week	**97±18**	**378±23**	**407±19**	**0.001***
P value**	**I vs. II=0.001**	**II vs. III=0.07**	**I vs. III =0.001**	
At the 20th week	**96.6±17**	**407±19**	**97.3±10**	**0.001***
P value**	**I vs. II=0.001**	**II vs. III=0.001**	**I vs. III =0.9**	
HbA1C (%)	**3.68±0.09**	**8.25±0.88**	**3.93±0.08**	
P value**	**I vs. II=0.001**	**II vs. III=0.001**	**I vs. III ==0.999**	

Data: Mean± SD

* To examine the mean difference all groups, the ANOVA test was utilized.
*** A P value of less than 0.05 indicates statistical significance. The post-hoc test with Tukey correction was applied for pairwise comparison.

3. Novel object recognition test

3.1.1. Familiarization session (Fig.1A). The experimentation rats investigated the same two objects (left and right) for the same amount of time during the familiarization session. Statistical analysis revealed a small difference (P>0.05) in the amount of time used by any group to research the two identical items during the training session.

3.1.2. Test session (Fig.1B): The statistical analysis showed that, during the test session, all experimental groups explored novel objects more than familiar ones, with the exception of the diabetic group, where there was no significant difference (P<0.05) in the amount of time spent exploring familiar and novel objects.

3.1.3. Discrimination ratio (Fig.1C): Rats in the control and diabetic-metformin groups explored the new object for a longer period of time during the test session than they did the familiar object. With the diabetes group's DR decreasing from (0.24±0.039) in the control group to (-0.0466±0.015) (P < 0.05) in the diabetic group, the capacity to distinguish between familiar and unfamiliar items was eliminated**.**

It is significant that the diabetic-metformin group's DR in novel object recognition tests exhibits a significant rise when contrasted with the diabetic group. An considerable impact of therapy on the DR was found using one-way ANOVA.

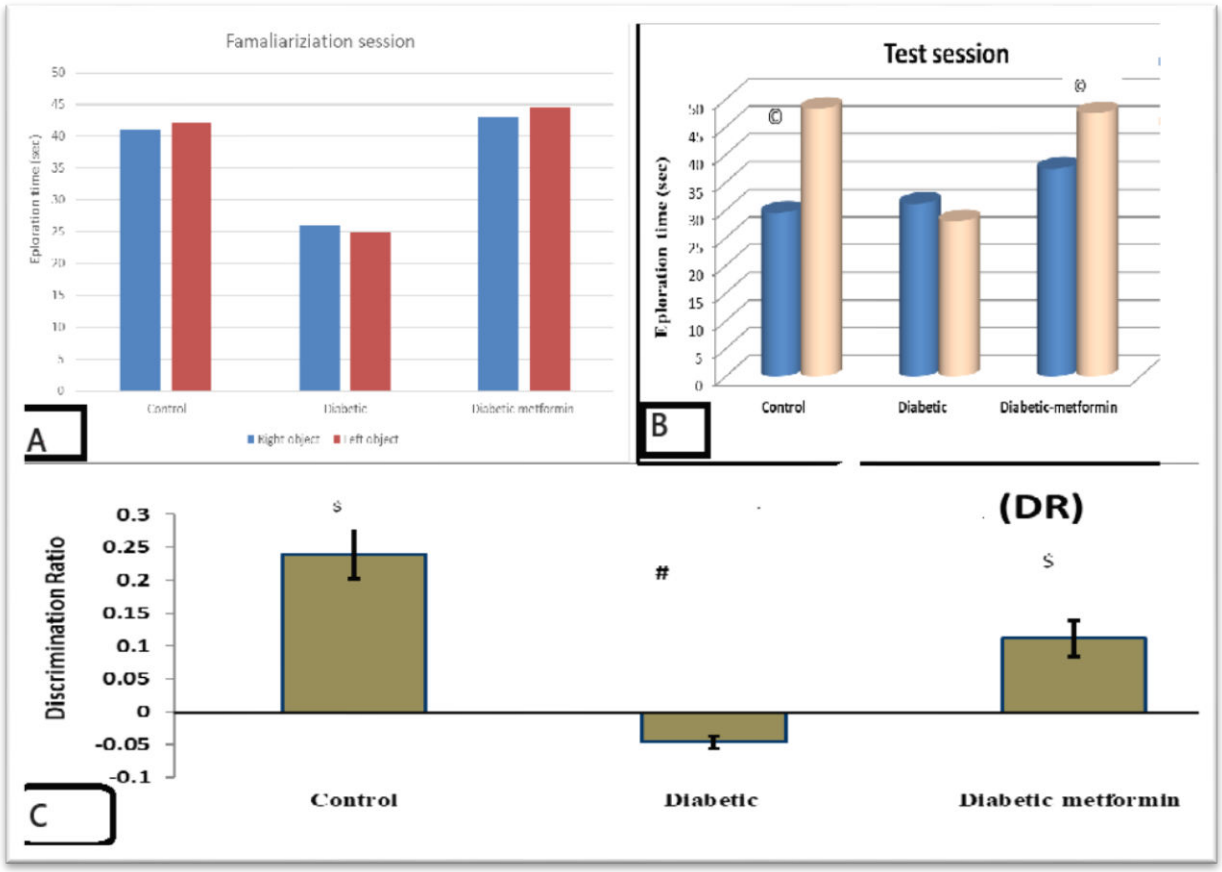

Fig.1. Representative Figures of novel object recognition test. A: showing the mean value of exploration time in familiarization session in different experimental groups. Data were represented

as mean ±SD. Data were analyzed by Paired -Samples T test. (N=6 rats per group). **B: showing the mean value of exploration time in test session in different experimental groups.** The data was shown as mean ±SD. The Paired-Samples T-test was used to evaluate the data. Six rats in each group. ©: When contrasting the novel with the familiar object, $P < 0.05$ was significant. **C: showing the mean value of discrimination ratio (DR) in different experimental groups**. The data was shown as mean ±SD. One-way ANOVA and post hoc Tukey tests were used to evaluate the data. Six rats in each group. $^{\#}$: $P < 0.05$ was significant when compared to control; $^{\$}$: $P < 0.05$ was significant when compared to diabetic.

4. T maze test (Spontaneous Alternation) (Fig. 2): Rats with diabetes showed memory impairments in T-Maze as compared to negative control groups; this was demonstrated by the diabetic group's significant decrease in alternation score, which went from 80% in the control group to 37% in diabetic group. When compared to untreated diabetic rats, the alternation score in the T-maze was higher in rats treated with metformin ($p < 0.001$)**.**

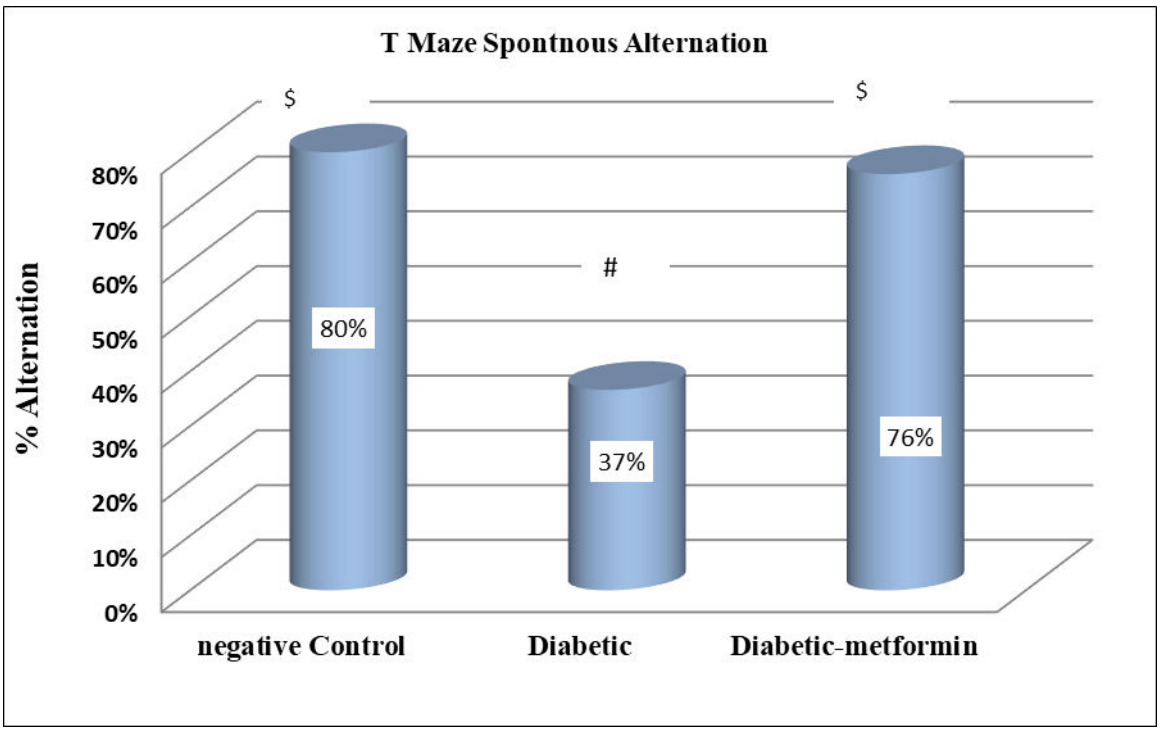

Fig. 2. showing the mean value of alternation score (%) in different experimental groups. The data was shown as mean ±SD. One-way ANOVA and post hoc Tukey tests were used to evaluate the data. Six rats in each group. #: $P < 0.05$ was significant when compared to control; $^{\$}$: $P < 0.05$ was significant when compared to diabetic.

5. Biochemical results

In this work, we evaluated TNF α and IL1β (a marker of inflammation) to identify the underlying mechanisms responsible for these T2DM-associated cognitive deficits and the mechanisms causing the positive effects of the diabetic-metformin group.

5.1. TNF-α level in hippocampus (Fig.3): The TNF-α level in hippocampal tissue was significantly higher in diabetic rats (753.2 ± 68 pg/mg tissue protein) compared to the control group (119 ± 21 pg/mg tissue protein) ($p = 0.001$), indicating enhanced hippocampal inflammation due to T2DM. It's noting that the concentrations of TNF-α in the diabetic-metformin group was significantly decreased compared to the diabetic group (P=0.001).

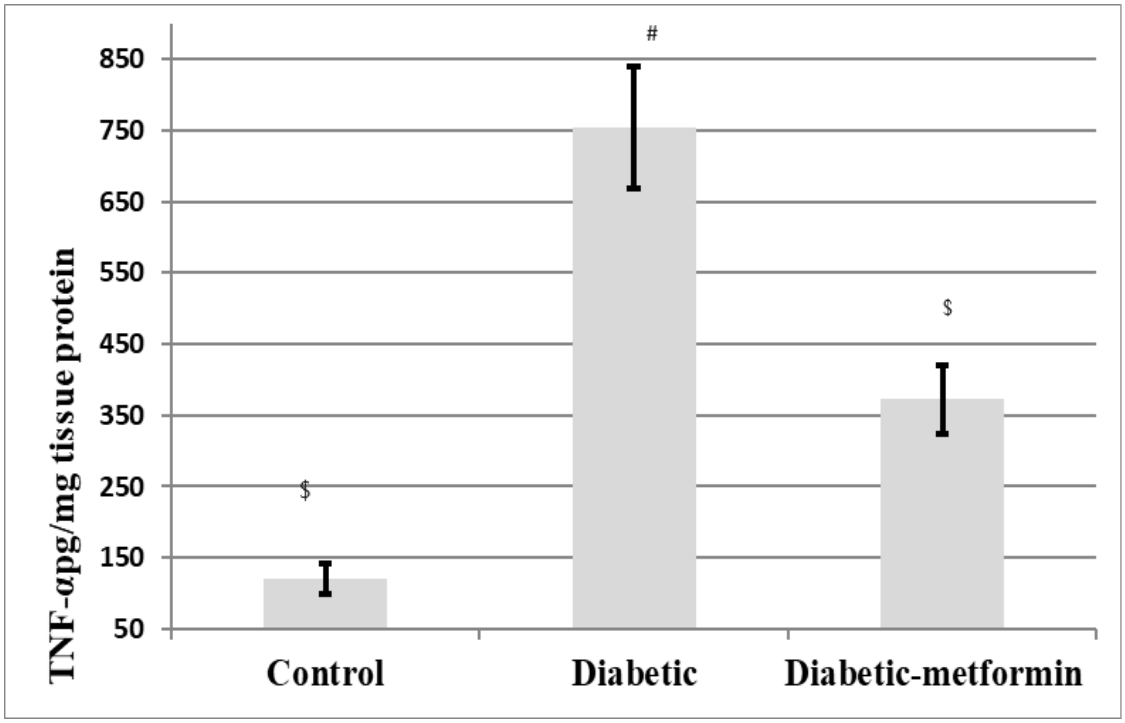

Fig.3. showing the mean value of TNF-α (pg/mg tissue protein) in the hippocampal tissue in different experimental groups. The data was shown as mean ±SD. One-way ANOVA and post hoc Tukey tests were used to evaluate the data. Six rats in each group. $^{\#}$: $P < 0.05$ was significant when compared to control $^{\$}$: $P < 0.05$ was significant when compared to diabetic.

5.2. IL-1β level in hippocampus : As seen in **(Fig. 4)**, T2DM increased hippocampal inflammation and the level of IL-1β in the hippocampal tissue increased significantly in diabetic rats to (455±43 pg/mg tissue protein) from (64±11 pg/mg tissue protein) in the control group. It is significant that, in comparison to the diabetes group, the IL-1β concentrations in the diabetic-metformin group were significantly decreased. These findings suggested that metformin may have anti-inflammatory properties in the diabetic rat's hippocampal tissues.

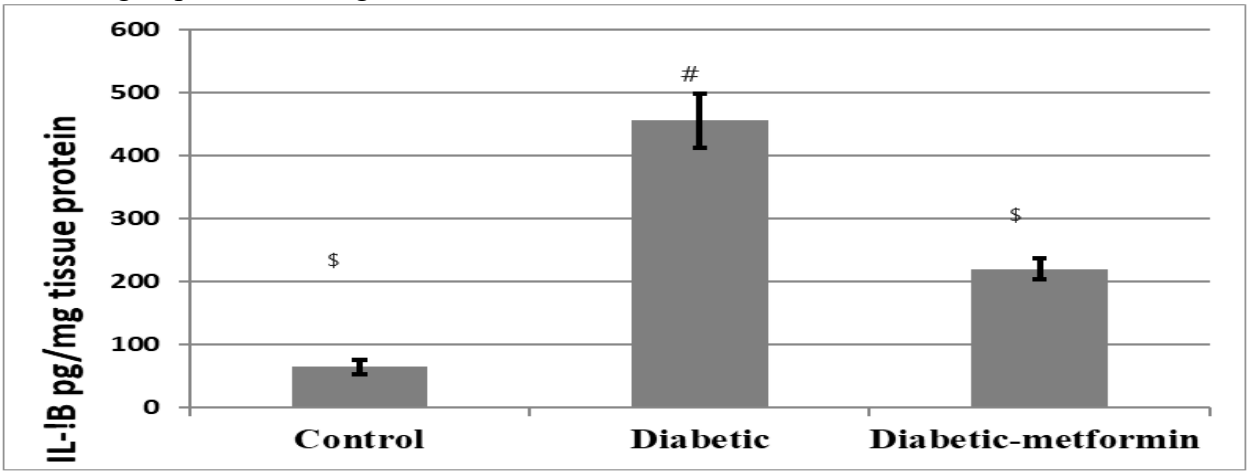

Fig.4. showing the mean value of IL-1B (pg/mg tissue protein) in the hippocampal tissue in different experimental groups. Data were represented as mean ±SD. Data were analyzed by one way

ANOVA test, Post hoc Tukey test. (N=6 rats per group). #: P < 0.05 was significant when compared to control $: P < 0.05 was significant when compared to diabetic.

6.Histopathological and Immunohisto-chemical results

6.1. General histological examination (Fig.5): Histological examination of hippocampus from control rats **(Fig.5A)** revealed the well-defined three layers of hippocampus; molecular layer (M), pyramidal cell layer (PC) and polymorphic layer (PM). PC layer has large neurons with basophilic cytoplasm and rounded vesicular nuclei with prominent nucleoli. The M and PM layers contain glial cells and normally apparent blood vessels. While figure from diabetic rat **(Fig.5B)** showed abnormal morphologies in the form of shrunken neurons with darkened, hyperchromatic, apoptotic nuclear changes, and even missing/lost neurons. Surprisingly, metformin partially attenuated such neuronal damages reported in diabetic rats **(Fig. 5C).**

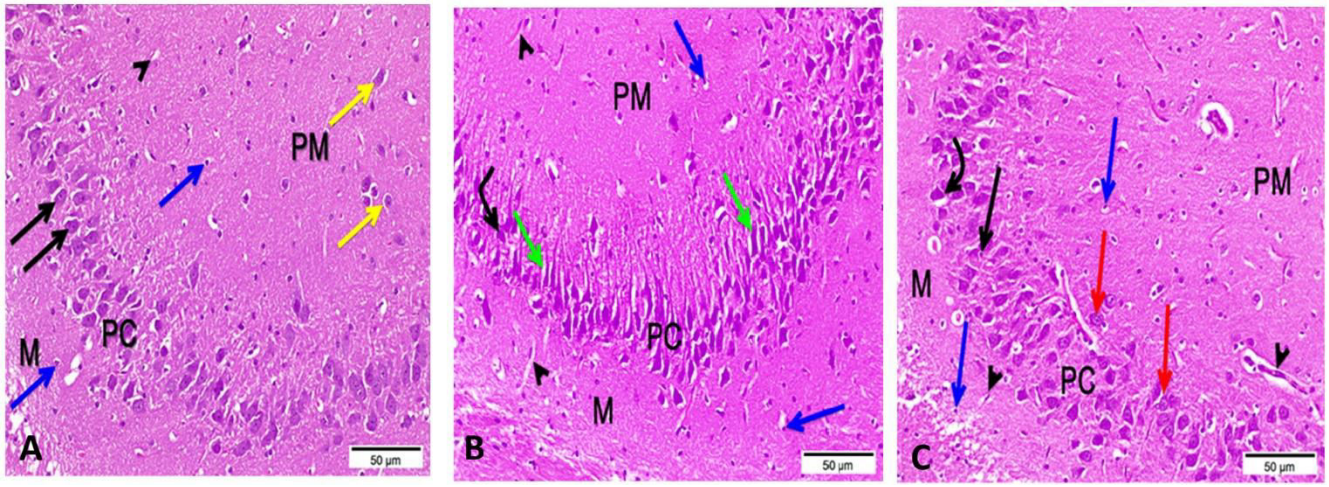

Fig. 5. Representative figures of the CA3region of the hippocampus stained with hematoxylin and eosin. Figure from control rat (A) showing well-defined three layers; molecular layer (M), pyramidal cell layer (PC) and polymorphic layer (PM). PC layer has large neurons (black arrow) with basophilic cytoplasm and rounded vesicular nuclei with prominent nucleoli. The M and PM layers contain glial cells (blue arrow) and normally apparent blood vessels (arrowhead). The PM layer also contains neurons of different sizes (yellow arrow). Figure from diabetic rat (B) showing well-defined three layers; molecular layer (M), pyramidal cell layer (PC) and polymorphic layer (PM). In PC layer, many neurons are deeply stained with darkly stained nuclei (curved arrow) and others are shrunken with pyknotic nuclei and wide surrounding spaces (green arrow). Blood vessels (arrowheads) and glial cells with widen surrounding spaces (blue arrows) are noticed in M and PM layers. Figure from metformin treated rats (C) in which the pyramidal cell layer shows few deeply stained neurons with wide surrounding vacuolization (curved arrow) and few degenerated neurons with pyknotic nuclei (green arrow). Most of neurons are normally looking with basophilic cytoplasm with rounded vesicular nuclei (red arrow). The molecular and polymorphic cell layers contain many glial cells (blue arrow) and blood vessels (arrowhead). Scale bar = 50 µm.

The alterations of histological appearance in the hippocampus of the three groups were summarized in (**Fig.6)** where there was significant decrease in the pathological grading induced by diabetes after treatment with metformin.

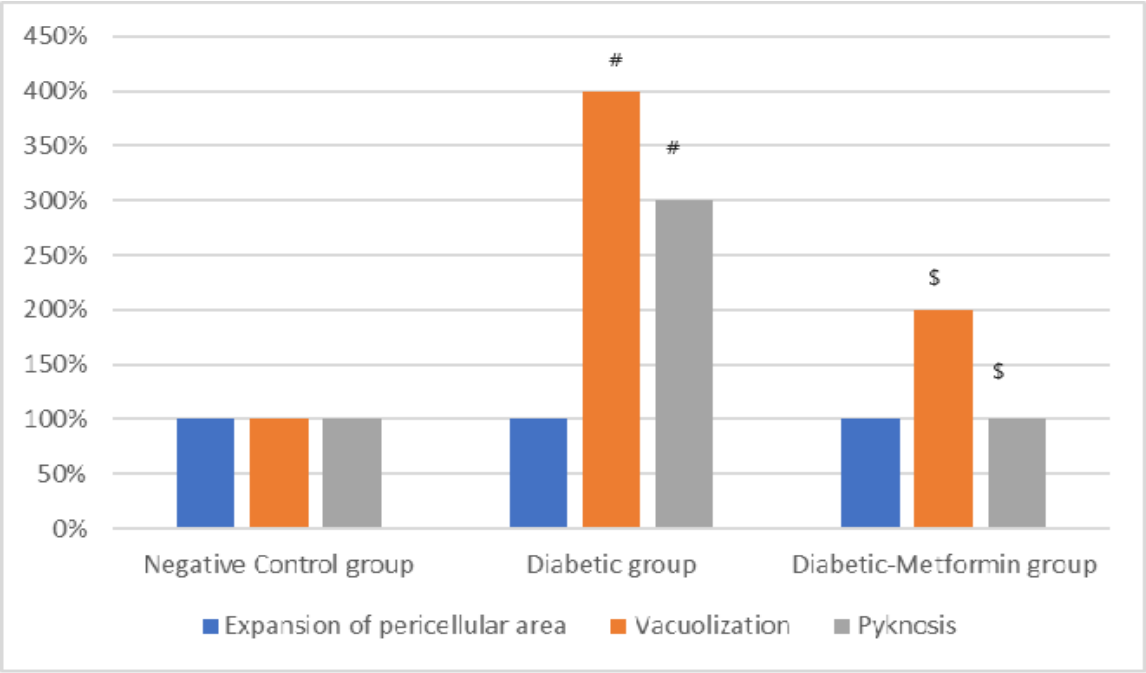

Fig.6. showing histopathological scoring of the hippocampus tissue. [#]: significant when compared to control, [$]: significant when compared to diabetic.

6.2. T2DM -Induced Apoptosis in the hippocampus

We evaluated the expression of the anti-apoptotic protein Bcl-2 and the apoptotic protein Bax in the CA3 region of the hippocampus to investigate the impact of metformin on T2DM-induced apoptosis.

6.2.1. Immunohistochemical expression of Bax protein (Fig.7)

The expression of Bax protein was week in the control group **(Fig.7A)**, high in diabetic group **(Fig.7B)** and surprisingly, moderate in diabetic-metformin group **(Fig.7C).**

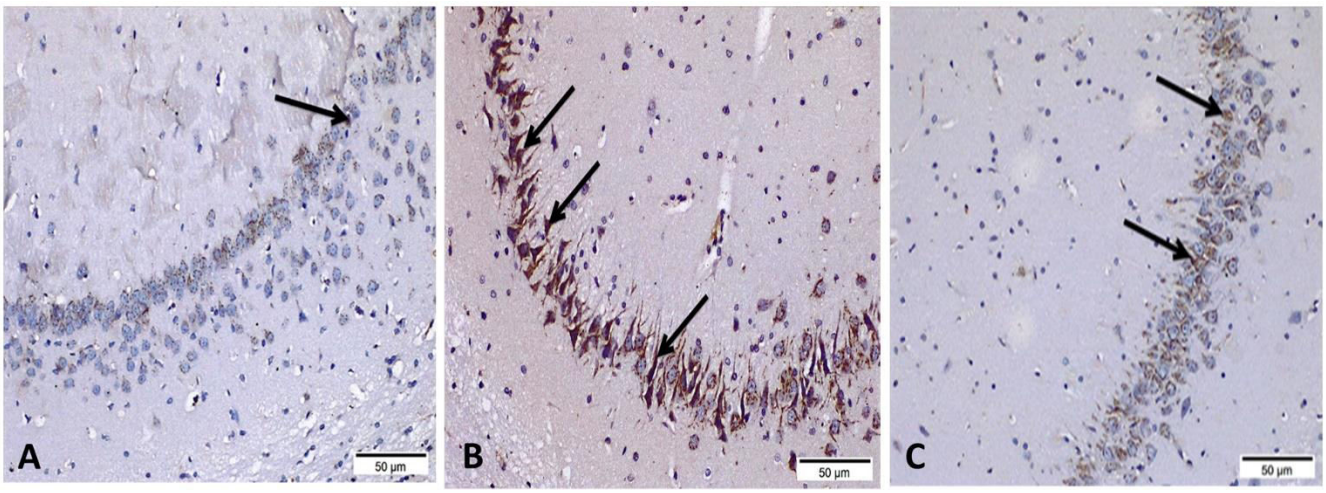

Fig.7. Immunohistochemical localization of proapoptotic marker Bax protein in hippocampus of all groups presented by brown color (arrows). Figure from control rat (A) showing faint immunostaining while in diabetic rat, the reaction is strong positive (B). On the other hand, figure from metformin treated rats showing mild positive reaction (C). Scale bar = 50 µm.

6.2.2. Immunohistochemical expression of Bcl2 protein (Fig. 8): The expression of Bcl2 protein was strong in the control group **(Fig.8A)**, week in diabetic group **(Fig.8B)** and surprisingly, moderate in diabetic-metformin group **(Fig.8C).**

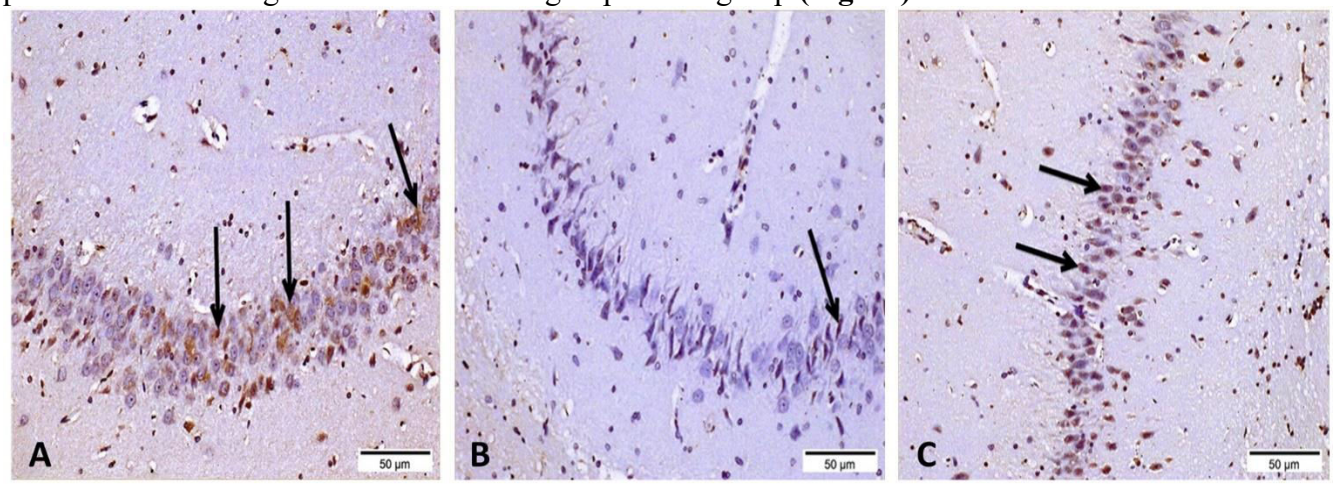

Fig. 8. Immunohistochemical localization of anti-apoptotic marker Bcl2 protein in hippocampus of all groups presented by brown color (arrows). Figure from control rat (A) showing strong positive reaction while in diabetic rat, the reaction is faint (B). On the other hand, figure from metformin treated rats showing mild positive reaction (C). Scale bar = 50 µm.

6.2.3. Immunoscoring:

6.2.3.1. Immunoscoring of Bcl2 (Fig.9): Bcl2 immunoexpression was significantly decreased in diabetic rats (P=0.001) compared to the control group. While in the diabetic-metformin group, it was significantly increased compared to the diabetic group (P=0.001).

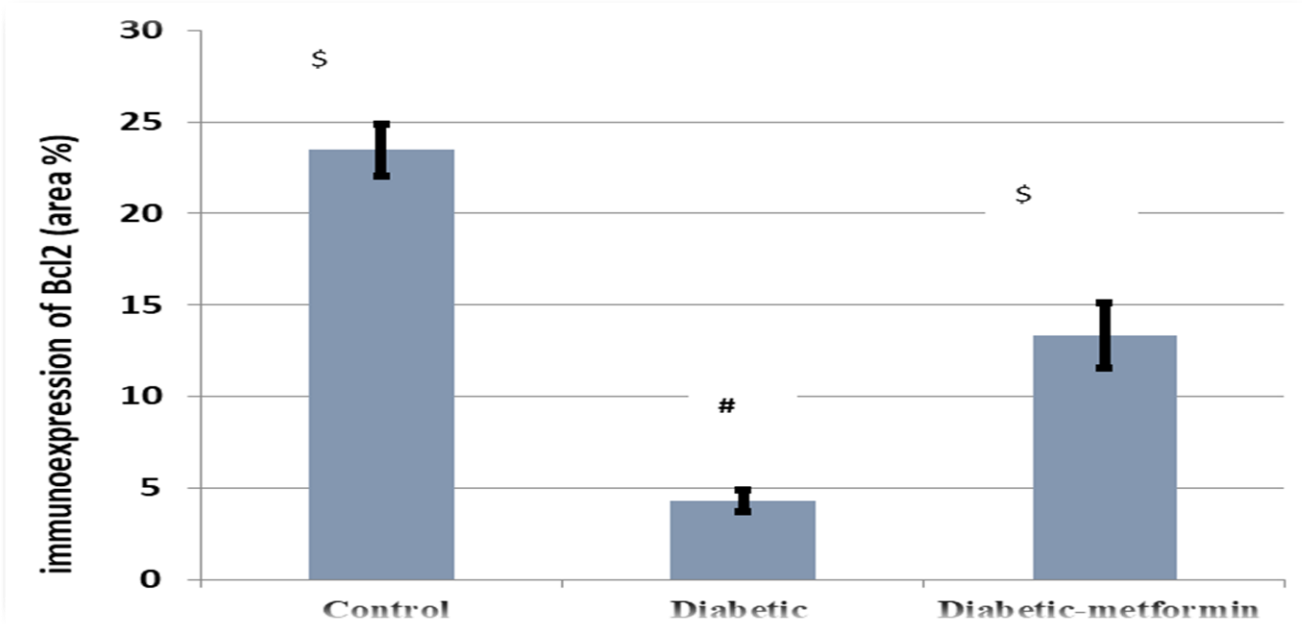

Fig.9. showing the mean value of immune-expression of BCl2 (area%) in the hippocampal tissue in different experimental groups. Data were represented as mean ±SD. Data were analyzed by one way ANOVA test, Post hoc Tukey test. (N=6 rats per group). $^{\#}$: $P < 0.05$ was significant when compared to control $^{\$}$: $P < 0.05$ was significant when compared to diabetic.

6.2.3.2. Immunoscoring of Bax (Fig.10): Compared to the control group, diabetic rats exhibited a significant rise in Bax immunoexpression (P=0.001). In contrast to the diabetic group, it was significantly lower in the metformin-diabetic group (P=0.001).

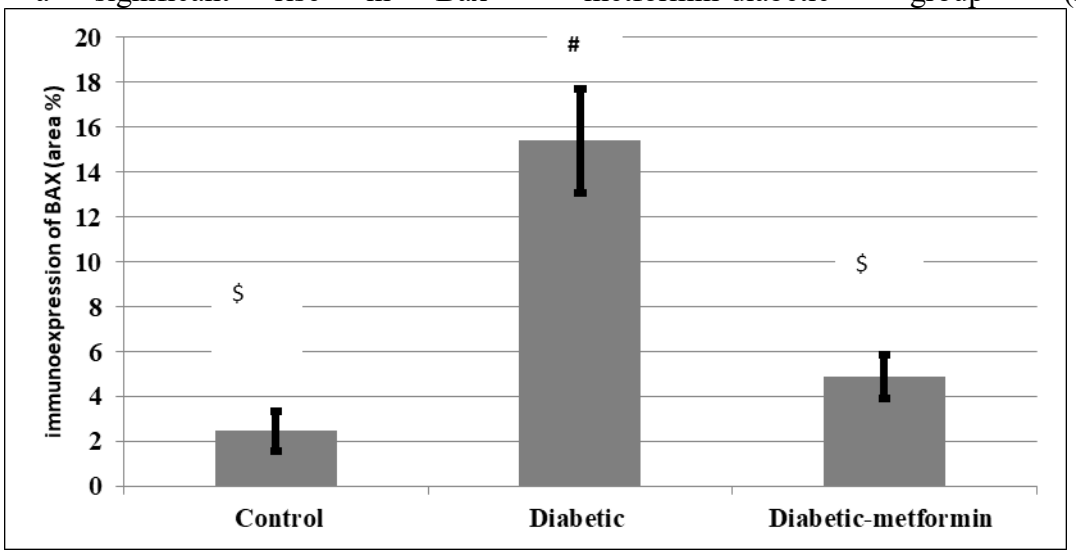

Fig.10. showing the mean value of immunoexpression of BAX in the hippocampal tissue in different experimental groups. Data were represented as mean ±SD. Data were analyzed by one way ANOVA test, Post hoc Tukey test. (N=6 rats per group). #: P < 0.05 was significant when compared to control $: P < 0.05 was significant when compared to diabetic.

6.2.3.3. Immunoscoring of Bcl2 / Bax ratio (Fig.11): There was a significant difference in the mean value of Bcl2/Bax ratio in hippocampus tissue between control group, diabetic group and diabetic-metformin treated group (P<0.001) as shown in (**Fig.13)**. T2DM enhanced hippocampal apoptosis, and Bcl2/ Bax ratio in hippocampus tissue was significantly increased in diabetic rats to (**356.56± 63.5**) from **(10.6± 3.76)** in the control group**.** It's noting that the Bcl2/ Bax ratio in the diabetic-metformin group was significantly decreased compared to the diabetic group (P<0.001).

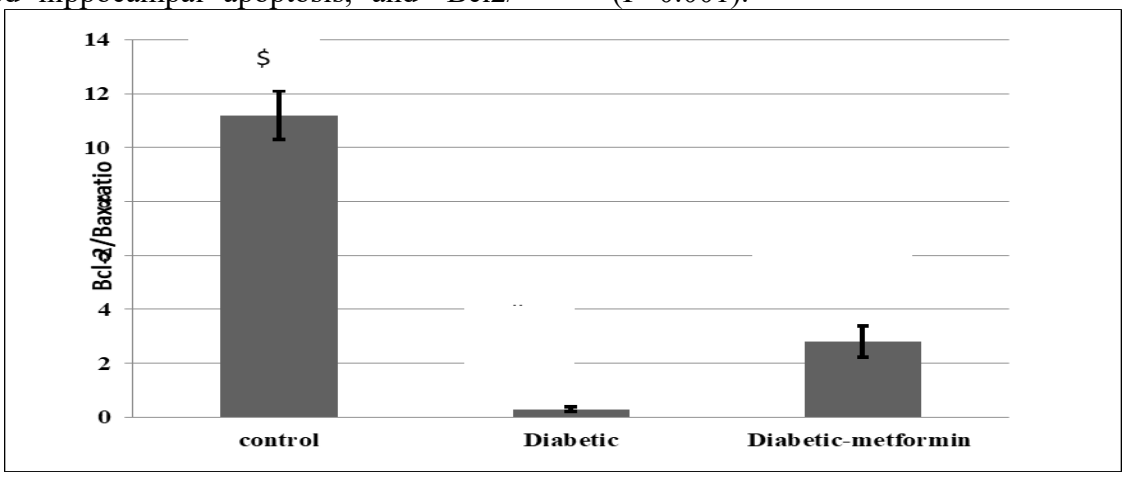

Fig. 11. showing the mean value of Bcl-2/Bax ratio in the hippocampal tissue in different experimental groups. Data were represented as mean ±SD. Data were analyzed by one way ANOVA test, Post hoc Tukey test. (N=6 rats per group). #: P < 0.05 was significant when compared to control $: P < 0.05 was significant when compared to diabetic.

Discussion

The study designed to detect the cognitive impairment induced in T2DM experimental rat model and assessing the role of metformin in improving these pathophysiological changes.

In the current study, we reported that using metformin for 10 weeks decreased blood glucose levels and body weight when compared to the diabetic group. Our findings corroborated those of previous studies, using metformin at a dose of 25 mg kg in diabetic rats resulted in greater body weight loss than the non- treated group **(Yanardag et al., 2021 and Matthaei et al., 2000). Viollet et al.** explained that Metformin treatment-related weight reduction was mostly ascribed to two pathways; insulin binds to the insulin receptor and metformin operates through 5′-AMP-activated protein kinase (AMPK). Both pathways increase the absorption of glucose regulating the energy expenditure of adipose tissue **(Viollet et al., 2007).**

One of the metabolic mechanisms that promotes free fatty acid oxidation, prevents the synthesis of free fatty acids and lipolysis in adipose tissue, signals the hypothalamus to increase intake of food, and increases the uptake of glucose by tissues is AMPK **(Wang, et al., 2021**).

In the present study, type 2 diabetic rats exhibited a significant increase in HbA1c and in diabetic-metformin group, HbA1c showed statistically significant improvement when compared to the diabetic group, this was supported by previous results of **(Schweizer, et al., 2007)** and **(Beysel, et al., 2018)**.

In the present study, diabetic rats exhibited spatial learning and memory impairments as confirmed by behavioral parameters in T maze and novel object recognition. In the previous results designed by **Gaspar et al., they** clarified that diabetes causes cognitive decline by causing neurobehavioral changes in diabetic animals, markedly elevated acetylcholine esterase activity, lowered glutathione levels, and activation of microglia and astrocytes with a corresponding decrease in hippocampal neuronal density **(Gaspar et al., 2016)**. This confirms our results where diabetes exhibited spatial learning and memory impairments as confirmed by behavioral parameters in T maze and novel object recognition in diabetic rats.

As recorded in the present work, **Cassano et al.** found that HFD/STZ rats had a significant reduction of DR in the NOR test in comparison to the control group, suggesting a working memory impairment. In diabetic-metformin group, the learning and memory impairments showed statistically significant improvement when compared to the diabetic group suggesting that the function of some neurons is restored **(Cassano et al.,2020)** Metformin was reported to lessen cognitive dysfunction in T2DM patients according to earlier research **(Ng et al., 2014**). There exist multiple plausible processes that elucidate this phenomenon. Metformin lowers insulin levels, improves inflammation and thrombosis, and lowers the risk of metabolic syndrome, according to studies. Additionally, it raises insulin sensitivity, which may guard against cognitive impairment **(Fahed et al., 2022**). **Zhou et al. findings** supported our records as metformin attenuates cognitive impairments in rats by of the T maze and novel object recognition tests results**.**

In the current study, we found that the diabetes group's hippocampal pro-inflammatory cytokine TNF-α level significantly increased than did the control group. The present findings corroborate previous findings showing, in this diabetic model, brain inflammation is one of the variables that contributes to cognitive impairment. **Rahmati et al.'s** study, which showed a considerable rise in pro-inflammatory cytokine expression (TNF-α and IL-1β) in the hippocampal regions of STZ-induced diabetic rats, provided evidence in favor of this (**Rahmati et al., 2021).**

Interestingly, our findings show that metformin significantly lowers the protein level of the pro-inflammatory factor TNF-α

in the hippocampal regions of diabetic rats. These findings are in line with earlier research showing that metformin inhibits microglia activation and the expression of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 at both the mRNA and protein levels (**Tao et al., 2018 and Zhou et al., 2018**). On the other hand, in a similar study performed by **Shukla, et al 2017),** they found that there was non- significant difference in levels of protein expressions of IL-1β and TNF-α.

The findings in the current study support the theory that DM might significantly worsen cognitive function and this could be explained by activating hippocampal neuronal cell pro-inflammatory and pro-apoptotic pathways. This is in consistence with the earlier findings of **Zhang et al., 2019** on diabetic mice as regard hippocampal neuronal disorganization and apoptosis.

In our study, metformin given to T2DM rats, dramatically improved hippocampus neuron survival. This may be explained by direct beneficial effects of metformin through anti-apoptotic and anti-inflammatory mechanisms that greatly enhance the hippocampal capacity for learning and memory. This result is in agreement with findings of studies performed by **(Ebrahimi et al., 2022**) and (**Akinola et al., 2022**).

Pervious study from **Hockenbery et al.** has been demonstrated that Bcl-2 inhibits apoptosis and prolongs cell viability, whereas Bax is Bcl-2's pro-apoptotic antagonist **(Hockenbery et al., 1990**).

By using immunohistochemistry, we showed that the expression of pro-apoptotic Bax protein in the hippocampus was significantly increased in diabetic rats compared to control and the expression of Bcl2 protein was significantly decreased in diabetic rats compared to control, which contributed to the apoptosis of hippocampus neurons so metformin decreases apoptosis.

Our results were consistent with the previous observations of **Wang et al.,** which demonstrated that fluctuating hyperglycemia in diabetic rat causes more severe inflammatory damage to the hippocampus than acute hyperglycemia, promotes neuron apoptosis, and impairs hippocampus function by Bcl-2/Bax signaling **(Wang et al., 2021**).

This study evaluated the neuroprotective effects of metformin on the expression of apoptosis-related proteins such BAX and BCL2 in the hippocampus. When comparing the diabetic-metformin group to the corresponding untreated diabetic group, metformin significantly decreased the Bax protein levels, and when comparing the diabetic-metformin group to the corresponding untreated diabetic group, it significantly increased the Bcl2 protein levels. Consequently, the BCL2/BAX ratio showed a significant decrease in the diabetic-metformin groups when compared to the corresponding untreated diabetic group.

The findings of this investigation are consistent with those of El Kiki et al. which indicate an increased Bcl2 /Bax ratio were found in rats who received metformin (El Kiki et al., 2020). Metformin was also reported to up-regulate the gene expression of Bcl-2 and down-regulate the gene expression of Bax and caspase-3 and hence modulated cognitive dysfunction **(Oda et al., 2017**).

Conclusion

Collectively, our results revealed the metformin exhibit a marked protection against diabetes-associated cognitive disorders and this effect was attributed to its anti-inflammatory and anti-apoptotic. Thus, these results may postulate a potential therapeutic role of metformin as a promising drug in the improvement of DM-induced cognitive dysfunction through reducing the hazardous pathophysiological effects that induced by DM on the hippocampus.

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