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Research Article

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The Efficacy of C–peptide on Impairment of Learning, Memory, and Apoptosis in Streptozotocin–induced Diabetic Rats Treated with Insulin

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ABSTRACT

Background and objectives: *Diabetes causes multiple long–term complications, including cognitive dysfunction that does not depend on the glycemic status of patients. Efficacy of C–peptide diabetic–related nephropathy has been previously investigated. In the present study, the sensitivity of diabetic rats to central streptozotocin (STZ) and amyloid–β peptide (Aβ) and the therapeutic effects of C–peptide were investigated.* ***Materials and methods:*** *Seventy–two male Sprague–Dawley rats (240 to 280 g) were randomly divided into nine groups: 1) control, 2) Aβ (2 µg/side intracerebroventricular [ICV] injection), 3) diabetic group, 4) diabetic + Aβ group, 5) STZ group (1.5 mg/kg ICV injection), 6) diabetic + STZ group, 7) diabetic + STZ + C–peptide (5 nmol/kg daily ICV injection), 8) diabetic + C–peptide group, and 9) high–dose STZ (3mg/kg ICV injection). All diabetic rats received daily subcutaneous 20 IU/kg insulin. Learning and memory were tested by Morris water maze on the 25th day and then the hippocampus was removed and the apoptosis indexes (caspase–3, Bax, and Bcl_2) were measured by western blot.* ***Results:*** *The present study revealed no difference in learning and memory impairment between diabetic and control group (P=0.51), but the spatial learning was significantly impaired in the group receiving high–dose central STZ (P<0.001). In addition, low doses of STZ caused impaired learning and memory in diabetic rats (P<0.001), prevented by C–peptide (P<0.001). The cleaved Caspase 3 and Bax/Bcl_2 ratio, increased in diabetic groups, was prevented with C–peptide (P=0.029).* ***Conclusion:*** *The results of the present study showed that impaired learning and memory in diabetic rats can be prevented by C–peptide.*

Keywords: *C–peptide; Diabetes Complications; Cognition Disorders; Hippocampus; Rats*

INTRODUCTION

Diabetes mellitus (DM) refers to a group of metabolic diseases characterized by hyperglycemia and is categorized into type 1 diabetes mellitus (T1DM) or insulin–dependent diabetes mellitus and type 2 diabetes mellitus (T2DM) or non–insulin–dependent diabetes mellitus (1). The high prevalence of DM has changed this condition to an important health problem, especially in southern cities of Iran (2).

Diabetes mellitus is associated with crucial long–term complications, including retinopathy, nephropathy, cardiovascular disorders, neuropathy, and cognitive dysfunction that increase the mortality rate of the affected

patients (3). These vascular complications have been contributed to hyperglycemia in patients with T2DM (4, 5) and various insulin treatment modalities have been proposed for their prevention (6, 7). However some studies have indicated that these complications are associated with other factors, rather than hyperglycemia, such as insulin resistance, depression, and smoking (8). Therefore, pure insulin therapy is not the most proper treatment for patients with T1DM (9).

The hypothesis that T1DM patients with remaining β–cell function are less prone to develop such complications (10) founded the introduction of C–peptide supplement in patients with T1DM; accordingly, co–treatment with insulin and C–peptide in patients with T1DM is found effective on renal function (11, 12), retinopathy (13), motor and sensory diabetic neuropathy (14, 15).

Diabetic encephalopathy is an important complication that causes cognitive dysfunction and is established to occur independent of glycemic status (16). In addition, Alzeihmer's disease is also associated with DM and hyperinsulinemia, because of the insulin–degrading enzyme (IDE) that degrades insulin and amyloid–β peptide (Aβ), which is found excessively in patient's brain with Alzeihmer's disease (17, 18). Animal studies were able to induce insulin resistance and cognitive dysfunction (deficit in spatial memory and learning), similar to human sporadic Alzeihmer's disease, by intracerebroventricular (ICV) administration of streptozotocin (STZ) (19, 20).

In the present study, we investigated spatial memory and learning impairments, induced by Aβ and STZ in different diabetic–induced rat groups and the efficiency of C–peptide on preventing these impairments.

Materials and methods

Study design

Seventy–two male Sprague–Dawley mature rats, weighing 240 to 280 g, were included in the current prospective study. All animals were housed individually and fed by standard food throughout the experiment. The animals were initially evaluated for illness by physical examination and laboratory screening and lived freely in cages (two rats per cage) with water and standard rodent chow. They were monitored and acclimated to the new environment for one week and were all housed under controlled standard laboratory conditions (21°C, relative humidity, and 12/12 hour photoperiod). In all stages of the study, the NIH guidelines and ethical considerations were met.

The animals were divided into nine study groups by simple randomization: 1) The control group received 2 µl/side ICV normal saline on the third day, 2) The Aβ group received 2 µg/2µl ICV injection by canulation, and Hamilton syringe 3) The diabetic group received 60 mg/kg STZ intravenously (10) on the first day and the serum blood sugar was measured on the third day. After confirmation of diabetes, 2 µl/side normal saline was injected in lateral ventricles and daily 20 IU/kg insulin was injected subcutaneously (SC),(4) The diabetic + Aβ received STZ for induction of diabetes, similar to the previously–mentioned protocol, and received 2 µg/µl Aβ in each lateral ventricles, 5) The STZ group received 1.5 mg/kg STZ in each lateral ventricle by Hamilton syringe on the first day, 6) The diabetic + STZ group, received IV STZ, similar to the previously–mentioned diabetes induction protocol, and then received 1.5 mg/kg STZ in each lateral ventricle and received daily SC 20 IU/kg insulin, 7) The diabetic + STZ + peptide C were diabetized and received ICV STZ, like the previous group, and also received 5 nmol/kg daily ICV injection of C–peptide and daily SC 20 IU/kg insulin, 8) The diabetic + peptide C group were induced with diabetes and received 5 nmol/kg daily ICV injection of C–peptide, 9) The high–dose STZ received 3 mg/kg STZ in each lateral ventricle on the 3rd and 5th day.

All groups underwent surgery on the 3rd day and cannulation was performed by stereotaxis method for ICV injection. The protocol of surgery was as follows: The animal was kept still, the needle was removed, and the injection needle (27 gauge) that was attached to a short poly–ethylene tube and 10 µl Hamilton syringe were placed inside the cannula. The needle was adjusted so that 0.5 mm of its tip be placed over cannula and right above the right or left ventricle to prevent brain damage. Then, STZ or Aβ was injected slowly during 5 minutes in lateral ventricles and after injection, the needle was not removed to prevent the return of the drug.

STZ and Aβ (Sigma Aldrich Company, USA) were diluted in normal saline and kept in -70°C freezer. Before injection, Aβ was incubated in 37°C for 72 hours.

The spatial learning and memory was tested by Morris water maze (MWM), performed for all groups on the 25^{th}–28^{th} day. This test analyzed the time to find the platform (escape latency), the distance traveled and the swimming speed by Ethovision software in two phases of hidden and visible platform. In a circular pool with 120 cm across and 50 cm height with 30 cm high water of 22°C, a hidden platform was placed 1.5 cm below the water surface at the southwest quarter and the animal was randomly released from each quarter and was allowed 90 seconds to find the platform. The animal's movements were filmed in darkness. If the animal could not find the platform, the researcher guided the rat to the platform and let the rat stay on it for 20 seconds. According to the protocol, the rats were trained for four days. After four experiments, the rat was dried with a towel and put back into the cage. In the next phase, a visible platform was placed over the water and the animal was released in another quarter to test its motor–sensorial system's provocation and function.

After the tests, the rats were sacrificed and the hippocampus was removed for measurement of apoptosis indexes (Caspase 3, Bax, and Bcl_2) by western blot and was kept in -70°C freezer.

Western blot

RIPA buffer was used for lysis of hippocampus tissue, then the samples were centrifuged at 14000 g for 10 minutes, the upper liquid containing protein was separated, the protein concentration was measured by Bradford solution using Biophotometer device (Eppendorf, Germany), and was analyzed by standard curves in Excel software. Then, 60 µg of samples were mixed with loading dye, were electrophoresized and blotted, and the proteins were transferred to PVDF paper in Mini–blotting device (BioRad, USA). The existence of protein was traced by chemiluminescency, Imaging XR^+ device (BioRad, USA) and analyzed by Image Lab software.

Statistical analysis

Data was analyzed using repeated measures two–way ANOVA and Benferroni post hoc test or one–way ANOVA and Tukey post hoc test. Results were presented as mean ±SEM. For the statistical analysis, the statistical software SPSS version 21.0 for windows (SPSS Inc., Chicago, IL) was used. P values of 0.05 or less were considered statistically significant.

Results

The mean escape latency of rats showed no significant difference in rats' function between diabetic and control groups (P=0.51) (Figure 1). Also, the studied dose of ICV Aβ had no effects on the control or diabetic groups (P=0.09) (Figure 2).

The spatial learning was significantly different between the control group and the groups receiving central STZ (P<0.001) (Figure 3). Also, there was a significant higher sensitivity to central STZ between diabetic and control groups (P<0.001) (Figure 4).

The swimming speed of rats was not different among the training days in the study groups (P=0.596) and the escape latency of visible platform was not significantly different among the training days in the study groups (P=0.408).

C–peptide could prevent the cognitive dysfunction, induced by low–dose STZ (ICV), in diabetic rats (P<0.001) (Figure 5). During apoptosis, the cleaved Caspase 3 and the Bax–Bcl_2 ratio increased. The amount of cleaved Caspase 3 was not significantly different among the study groups (P=0.07) (Figure 6). Also, Bax–Bcl_2 was not different between control and diabetic groups (with or without Aβ) (P=0.18) (Figure 7).

The different cleaved Caspase 3 between the control and diabetic groups (with or without low dose central STZ) indicated the effect of Aβ in the diabetic group induced with STZ (P=0.029) and C–peptide could prevent the destructive effects of STZ, regarding cleaved Caspase 3 (P<0. 05) (Figure 8).

Also, central injection of low–dose STZ could increase the Bax/Bcl_2 ratio in diabetic groups that could be prevented by C–peptide (P=006) (Figure 9).

Discussion

We found impaired spatial memory and learning in the group receiving high–dose central STZ (P<0.001), and low–dose STZ in diabetic rats that could be prevented by administration of C–peptide (P<0.001). The cleaved Caspase 3 and Bax/Bcl_2 ratio increased in diabetic groups that was prevented with C–peptide (P=0.029).

Sima and colleagues have compared 20 diabetic rats in two groups of with and without C–peptide (75 ng/kg for seven months) with 10 non–diabetic rats and have demonstrated that C–peptide could prevent spatial learning and memory deficit (21), which is consistent with the results of the present study. Biessels and coworkers investigated the effect of insulin treatment in STZ–diabetic induced rats and have suggested that insulin could not reverse the deficits in maze learning (22), which is in line with the results of the present study, as all rats received insulin, while treatment effects were only observed in groups receiving C–peptide. Francis and colleagues have proven the effect of intranasal insulin, but not subcutaneous insulin, on cognitive decline of STZ–induced diabetic rats (23), which is similar to the results of the present study regarding the inefficiency of SC insulin on learning impairment induced by diabetes.

Also, Sima and colleagues reported overexpression of caspase 3 and Bax that remained unchanged in C–peptide group and concluded that C–peptide could prevent hippocampal neuronal loss (21), which is in line with the results of the present study. Nevertheless, they have reported no difference in Bcl expression, while in the present study, Bcl_2 decreased significantly by C–peptide. As Sima and colleagues have hypothesized, efficiency of C–peptide underlies beneath the prevention of oxidative stress (21), which is of great importance in vascular complications of DM (24). In another study, Sima and colleagues have contributed the innate immune responses in diabetic encephalopathy and have posited that C–peptide prevents the inflammatory cascade in hippocampi of diabetic rats (25). Li and coworkers have further investigated human neuroblastoma SH–SY5Y cells and have reported synergistic effects for C–peptide with insulin on cell proliferation, and anti–apoptotic effects on high glucose–induced apoptosis (26). Other studies have also proven anti–apoptotic effects of C–peptide in other diabetes–related complications; Rasheed and colleagues have investigated the TNF–mediated cell death in proximal tubular kidney cells and have proven that C–peptide and insulin protect against diabetes–related tubular injury and apoptosis (27). Although, the study population and tissue sample was different in the above–mentioned studies than the present study, they have achieved similar results, regarding the efficiency of C–peptide. Further studies, investigating other crucial diabetes–related complications, might be able to indicate the beneficiary effect of C–peptide on all or most of diabetes–related complications, due to the common underlying mechanism for these complications.

In the present study, there was no significant difference in spatial learning and memory deficit between diabetic rats and the control group, which was possibly due to the duration–related apoptotic neuronal loss in rats, as described by Li and colleagues, who showed prolonged latencies in the MWM test in 8–month diabetic rats, but not in 2–month diabetic rats (28). Although, four weeks after diabetic induction in rats might not have been sufficient to induce cognitive dysfunction in the present study, the diabetic rats had increased sensitivity to central STZ; thus, based on the results of the current study, it is hypothesized that T1DM can increase brain's sensitivity to sporadic Alzeihmer's disease.

Another finding of the present study included the finding that one dose Aβ could not impair cognitive function, as there was no difference in the escape latencies of Aβ group, compared to the control group. As postulated, induction of cognitive dysfunction in rats by Aβ might require several injections and higher doses (29, 30). Yet, future studies may be able to indicate such effect.

The strengths of the present study include assessing a large number of rats in nine different groups that could clarify the effect of each intervention simultaneously. In addition, as far as the authors are concerned, the present study was the first study that investigated the efficiency of C–peptide on rats with induced cognitive dysfunction, while other studies have evaluated the changes in spatial learning and memory on diabetic rats without established brain complication. On the other hand, the present study included some limitations, including short follow–up of diabetic rats, which resulted in lack of difference in cognitive function of the diabetic group with the control group and longer follow–up is suggested in future studies. Although this limitation did not affect the results of the present

study, as cognitive dysfunction was successfully induced in rats (central STZ group) and the efficacy of C–peptide could be well demonstrated.

Conclusion

Learning and memory impairment in diabetic rats, induced by central STZ, can be prevented by C–peptide. Thus, C–peptide can be an important therapeutic agent in diabetic patients for prevention of this long–term diabetes–related complication. Future human studies can illustrate the efficiency of C–peptide on diabetic–induced learning and memory impairment.

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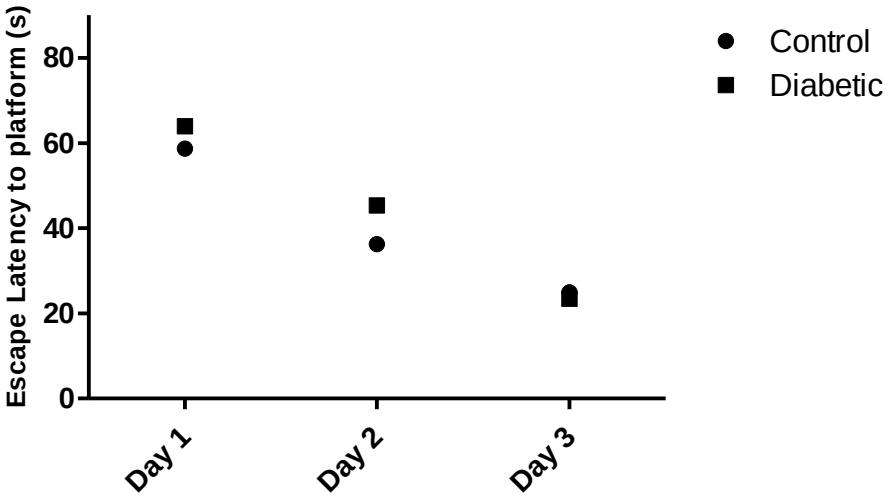

30.

Figure 1. The mean differences of escape latency between diabetic and the control group

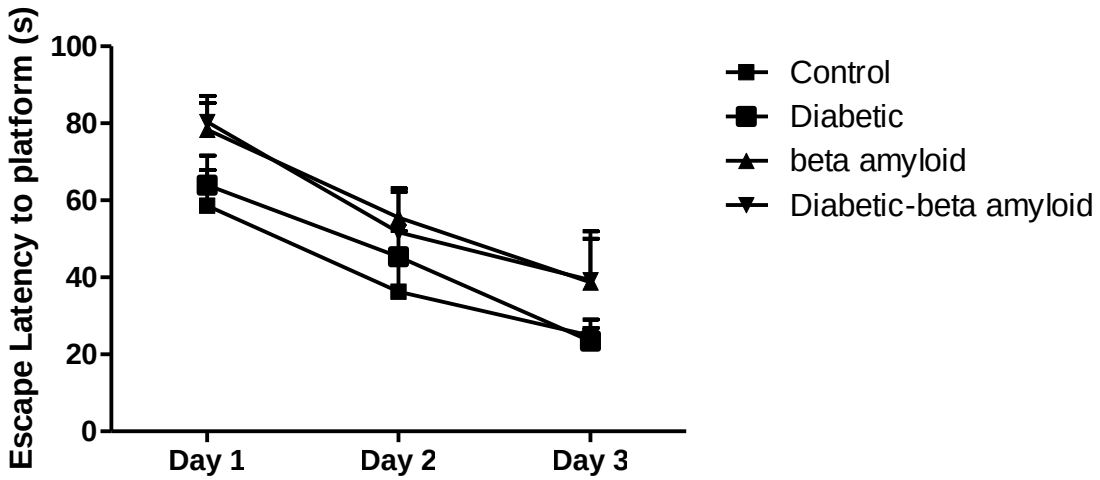

Figure 2. The effect of one dose amyloid–β on the escape latency of rats in the control and diabetic groups during three learning days

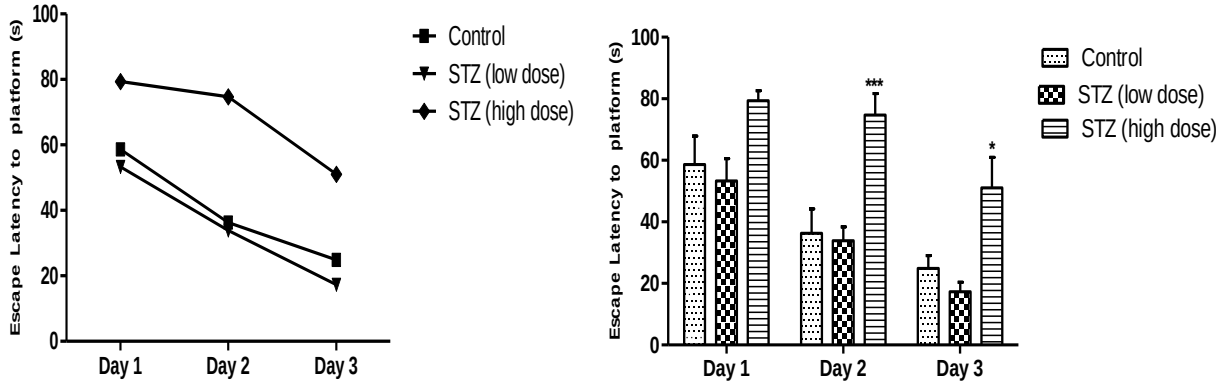

Figure 3. The learning pattern of rats in different study groups (left) and the differences of mean escape latency among the group receiving STZ (ICV) compared to the control group (right) (*P<0.05 and ***P<0.001 shows the difference between control group and the other groups)

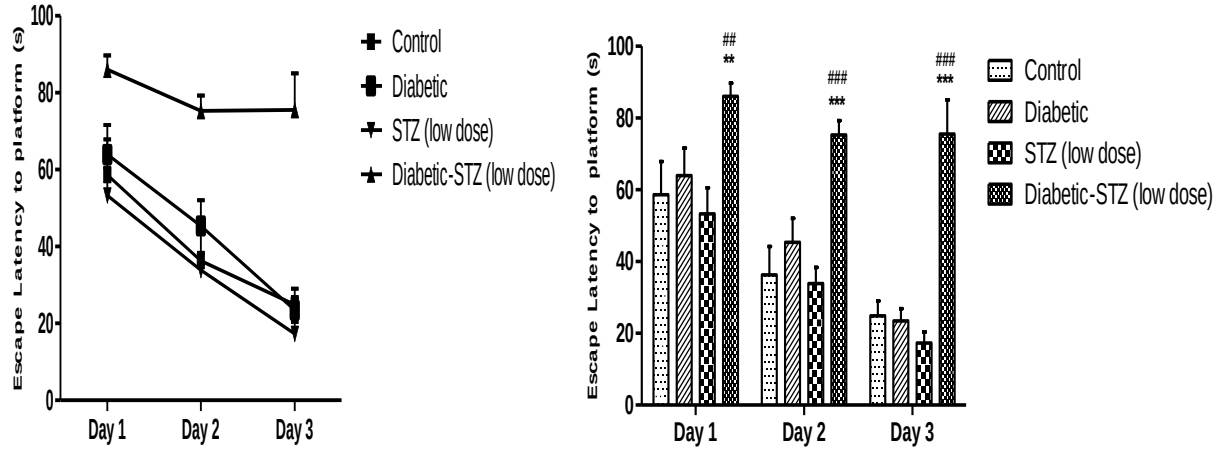

Figure 4. The learning pattern of rats in different study groups (left) and comparing the effect of low dose STZ between diabetic rats with the control group (right) (**P<0.01 and ***P<0.001 shows significant difference with the control group and ##P<0.01 and ###P<0.001 shows the difference between STZ (ICV) groups and STZ (ICV) + diabetic group)

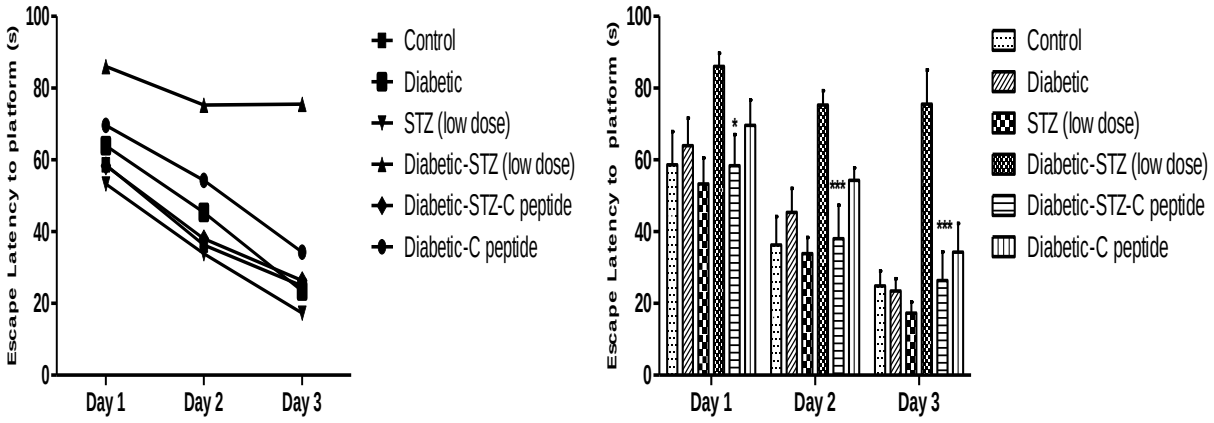

Figure 5. The learning pattern of rats in different study groups (left) and comparing the effect of C–peptide on memory disorder in STZ–induced diabetic rats with the control group (right) (*P<0.05 and ***P<0.001 shows the difference between diabetic + STZ (ICV) and diabetic + STZ (ICV) + C–peptide groups)

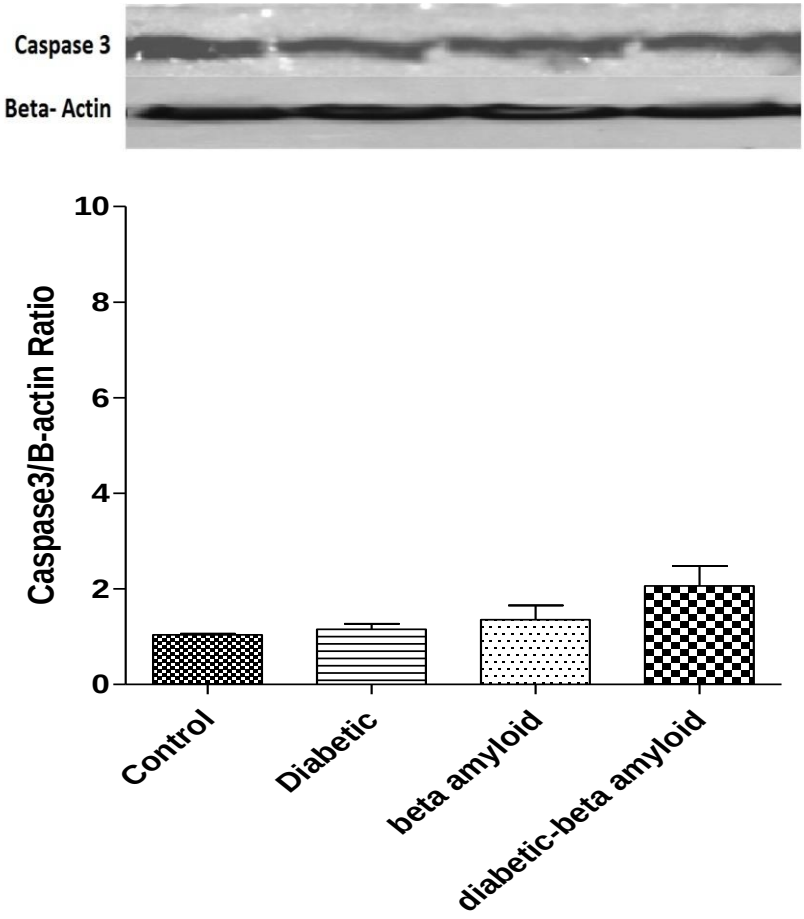

Figure 6. Western blot analysis, indicating the effect of amyloid–β on the cleaved Caspase 3 in rats' hippocampus

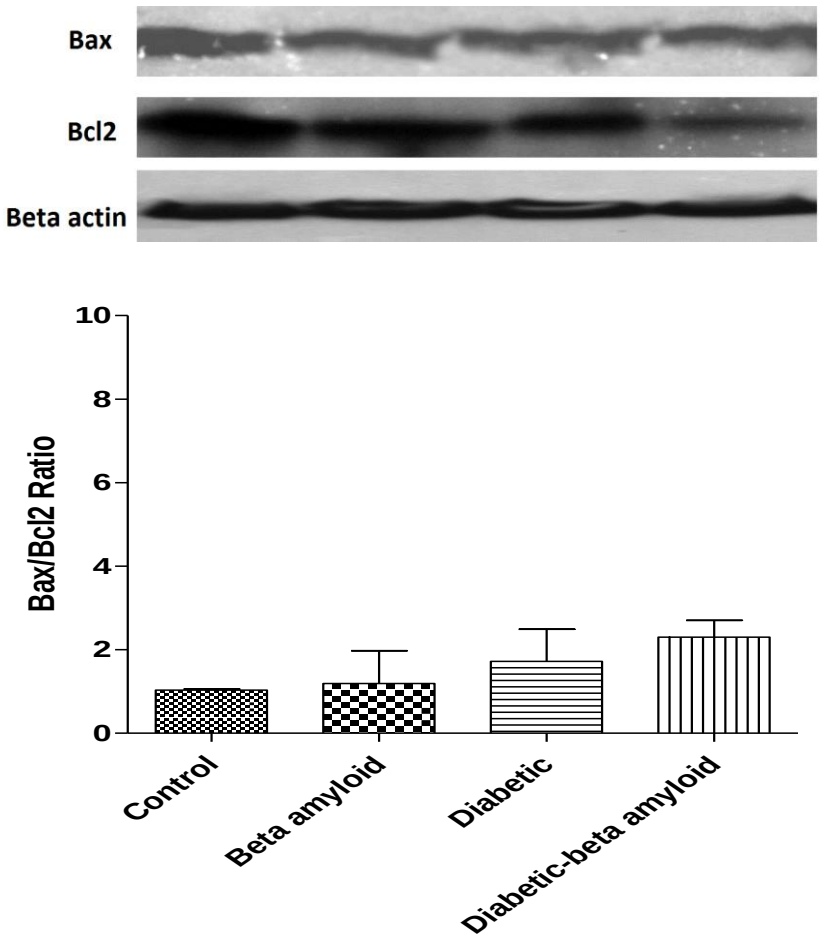

Figure 7. Western blot analysis, indicating the effect of amyloid–β and diabetes on Bax/Bcl_2 ratio in rats' hippocampus

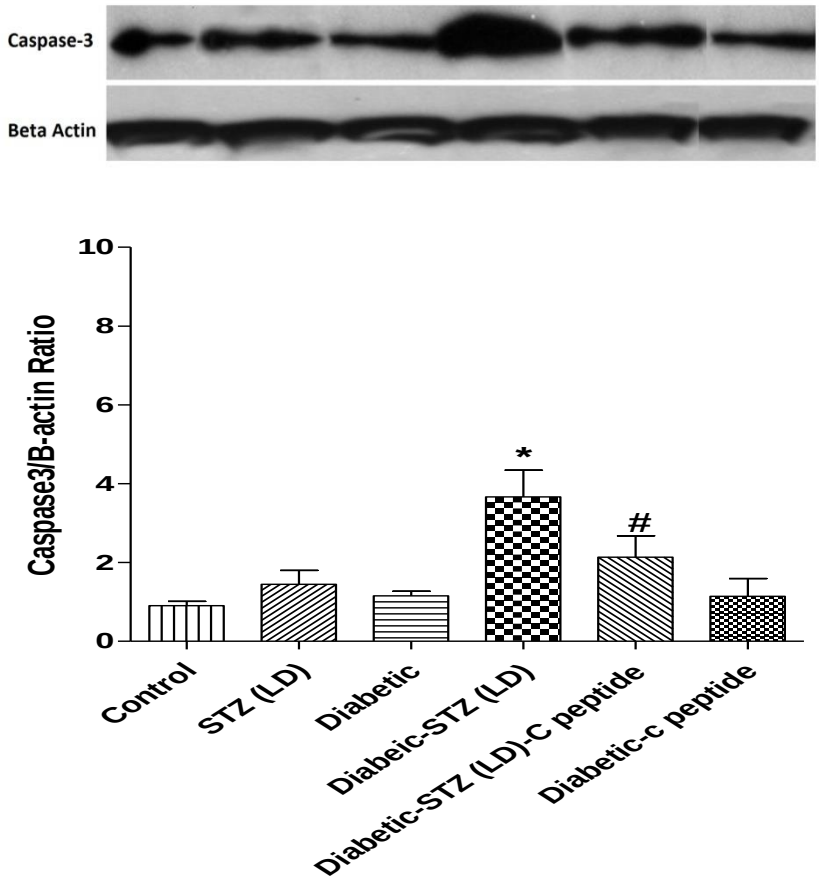

Figure 8. Western blot analysis, indicating the effect of central STZ on the cleaved Caspase 3 in rats' hippocampus (*P<0.05 shows the difference between control group and diabetic + STZ (ICV) group, and #P<0.05 shows the difference between diabetic + STZ (ICV) group and diabetic + STZ (ICV) + C–peptide group)

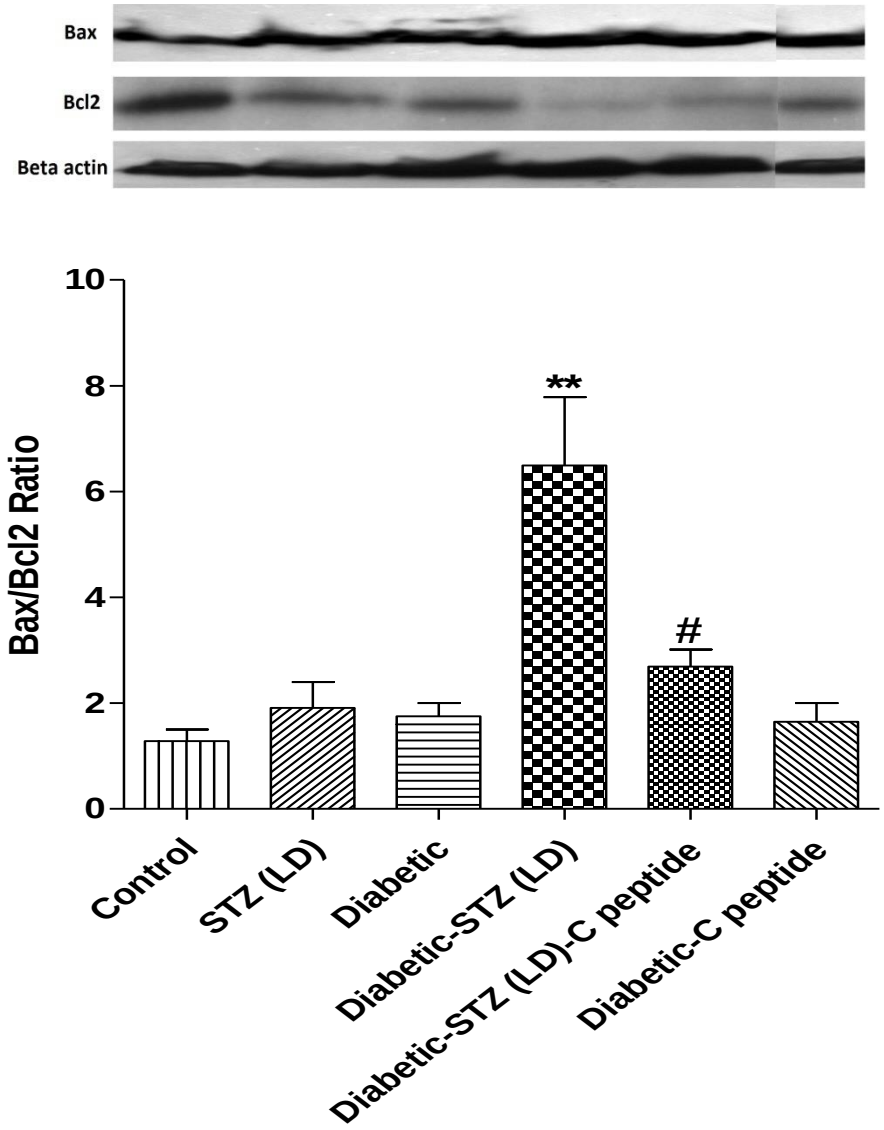

Figure 9. Western blot analysis, indicating the effect of C–peptide on Bax/Bcl_2 ratio in rats' hippocampus (**P<0.01 shows the difference between control group and diabetic + STZ (ICV) group, and #P<0.05 shows the difference between diabetic + STZ (ICV) group and diabetic + STZ (ICV) + C–peptide group)