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Comparing the acute and chronic effects of metformin and antioxidant protective effects of N-acetyl cysteine on memory retrieval and oxidative stress in rats with Alzheimer's disease

Niloufar Darbandi*, Samira Moghadasi, Hamid Reza Momeni and Matin Ramezani
Department of Biology, Faculty of Science, Arak University, Arak, Iran

Abstract: It has been suggested that oxidative stress plays an important role in neural degeneration and Alzheimer's disease. Some studies have shown that metformin has some beneficial effects on the brain and reduces oxidative stress, while others reveal that metformin increases oxidative stress in diabetic patients. In this study acute and chronic effects of metformin and antioxidant protective effects of N-acetyl cysteine in Alzheimeric rats were investigated. Animals were divided into seven groups (n=8): Control, STZ, STZ + metformin (one, three and eleven weeks), STZ+ metformin (eleven weeks) +N-acetyl cysteine (eleven weeks) and N-acetyl cysteine (eleven weeks). ICV injections of saline (1µl/rat) or STZ (3mg/kg) and IP injections of Saline (1ml/kg), metformin (200mg/kg) and/or N-acetyl cysteine (100mg/kg) were done. Memory retrieval, CA1 neurons density and serums oxidative stress were investigated. STZ injections reduced memory retention, intact neurons and increased serum oxidative stress compared to the control ($p<0/001$). Metformin injection for one and three weeks (but not eleven weeks) improved the effects of STZ ($p<0/001$). Administration of N-acetylcysteine with metformin (eleven weeks) improved STZ bad effects ($p<0/001$). It seems that acute and chronic consumption of metformin have different effects on memory retrieval, CA1 neurons and serum oxidative stress factors in AD rats.

Keywords: CA1 area, memory retrieval, metformin, N-acetyl cysteine, oxidative stress, rat, streptozotocin.

INTRODUCTION

Alzheimer's disease (AD) is specified by progressive neurodegenerative changes gradually reducing cognitive and functional abilities (Baker, 2011). Accumulating evidence suggests that AD is a metabolic disease during which brain loses its potential to utilize glucose efficiently. This is because producing energy will result in insulin resistance (Baker, 2011). Despite the fact that the mechanisms of AD is not fully understood yet, it is believed that it would be an age-related disease and enhanced by complex interactions between genetic and environmental risk factors (Laws, 2003). It has been suggested that oxidative stress, involving in the aging process and its damage has a major role to play in neuronal degeneration in Alzheimer's disease (Mangialasche, 2003).

The hippocampus, a part of the limbic system, is a major component of brain having a vital role to play in both long-term and spatial memories. Hippocampal deterioration is one of the earliest symptoms of the AD and significant feature of dementia is thought to be memory loss followed by multiple cognitive deficits (Hampel, 2008). The hippocampus, as whole, has the shape of a curved tube, includes the dentate gyrus (DG), a ram's horn or Cornu Ammonis, (CA1 through CA4) and the subiculum (Hampel, 2008).
Streptozotocin (STZ) is produced by the soil bacterium Streptomyces achromogenes (Sangi, 2019). Intracerebroventricular (ICV) administrations of STZ (sub diabetogenic dose, 3 mg/kg) in rodents are thought to be the good models for imitating sporadic AD pathology progression, which results in a considerable increase in the total tau protein and β-amyloid and a decrease in spatial cognition. Increasing the β-amyloid accumulation can raise the inflammatory process and free radical formation as well (Chen, 2014). These changes are accompanied by the reduction of GSK-3alpha/beta ratio (phosphorylated/total) in the brain (Plaschke, 2010).

Metformin has been used for many years to treat type 2 diabetes. The mechanisms behind that can reduce hepatic glucose output and increase the insulin-mediated absorption of glucose (He, 2009). Besides, it can rapidly pass through the blood-brain barrier (BBB) and has ample beneficial impacts on the brain such as promoting the antioxidant and anti-inflammatory properties, neuroprotective effects and neurogenesis which all mentioned can finally enhance the spatial memory (Rojas, 2013; Wang, 2012). Although in some studies it has been demonstrated that metformin reduces oxidative stress (Rojas, 2013; Cheraghi, 2014; Memisogullari, 2008), others reveal that the chronic use of metformin increases oxidative stress in the diabetic patients (Khouri, 2004; Pavlovic, 2000). It was showed that metformin may raise oxidative stress by increasing homocysteine levels which is associated with the generation of superoxide anions more and more and decreased activity of antioxidant enzymes (Pavlovic, 2000).

*Corresponding author: e-mail: N-Darbandi@araku.ac.ir

Antioxidants are the prominent groups of molecules that significantly postpone/ prevent the oxidative stress. These compounds scavenge reactive oxygen species (ROS) precursors. Moreover, they are responsible for the hindrance of ROS, and many free radicals in addition to the binding of metal ions which are necessary for the catalysis of ROS generation (Lappas, 2003). N-acetyl cysteine (NAC) accounts for a thiol group, acting as a potent antioxidant, ant-apoptotic and free-radical scavenger in which the serum homocysteine levels decrease and detoxification promote (Lappas, 2003). and finally increase the glutathione stimulates (Fulghesu, 2002). It is freely filterable with an easy access to the blood-brain barrier and intracellular compartments (Farre, 2004). NAC is able to modulate redox signaling, reduce lipid peroxidation and act as a substrate for the synthesis of GSH (Prakash, 2009).

According to the literature, it seems that acute and chronic consumptions of metformin have different effects on streptozotocin-induced learning and memory deficits (Asadbegia, 2016; Chung, 2015; Zhao, 2014) and oxidative stress (Cheraghi, 2014; Khouri, 2004; Pavlovic, 2000; Esteghamati, 2013; Yilmaz, 2005). Since diabetic patients are exposed to long-term metformin, it is essential to examine the long-term effects of this drug and the way it is combated. Therefore, the present study investigated the acute and chronic therapeutic effects of metformin and antioxidant protective effects of N-acetyl cysteine on memory retrieval, hippocampal CA1 neurons, and serum oxidative stress biomarkers in STZ-induced Alzheimer's disease.

MATERIALS AND METHODS

Animals' model

In our project Male Wistar rats (Pasteur Institute; Tehran, Iran) weighing 220-250g at the surgery time were utilized. The animals were kept in an animal home under a 12-h light/12-h dark cycle and they had been controlled in 22±2°C temperature. They had free access to food and water. They were allowed to be adapting to the conditions, for minimum one week before the surgery and were handled for 5 min/day during this adaptation period. Eight rats were put in each experimental group. All experiments were executed in accordance with the National Institutes of Health Guide for Care and use of Laboratory Animals (Publication number 85-23, revised 1985). Each rat was used only once. All behavioral experiments were done between 9:00 AM and 12:00 PM.

Treatment

All rats were randomly divided into seven groups. In control group, rats received ICV injections of saline (10µl for each lateral ventricle) under the stereotaxic surgery, on first and third days, and they were given intraperitoneal (IP) injections of saline (1ml/kg), every single day, during three weeks. In STZ group, rats received ICV injections of STZ (3mg/kg) for first and third days (Chen, 2014) and IP injections of saline (1ml/kg) for every day during three weeks. In other groups which were treated by metformin (one week, three weeks and eleven weeks), rats received ICV injections of STZ (3mg/kg) for first and third days and IP injections of metformin (200 mg/kg) (Zhao, 2014) during one, three or eleven weeks. In the group of STZ+ metformin+ N-acetyl cysteine, rats were given ICVinjections of STZ (3mg/kg) for the same days, IP injections of metformin (200mg/kg) and N-acetyl cysteine (100mg/kg) (Prakash, 2009) both over eleven weeks. Finally, in N-acetyl cysteine group, rats were given ICV injections of saline (10µl for each lateral ventricle), on the first and third days, and they also were given IP injections of N-acetyl cysteine (100mg/kg) for per day during eleven weeks.

Daily administrations were performed between 9:00 am and 12:00 pm. At the end of the treatments, the animals were trained into the step-through apparatus. One day (24h) later, a retention test was done to determine the ratio of long-term memory deficit. After doing the behavioral test, their brains were removed and stained with hematoxylin-eosin (H&E) to examine the impact of different treatments on hippocampal CA1 pyramidal neurons. In order to Biochemical analysis, blood samples, from the right ventricle of the heart, were processed to determine the level of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and Ferric Reducing Ability of Plasma (FRAP). Blood samples were centrifuged using a refrigerated centrifuge (universal device, made in Germany) at 4◦C (13,300 rpm for 10 min) and the supernatant was frozen at −20◦C in aliquots until using biochemical assays.

Drugs

The drugs used in the study were Streptozotocin (Sigma-Aldrich, USA), metformin (Arya, Tehran- Iran) and N-acetyl cysteine (Sigma-Aldrich, USA). The Drugs were dissolved in sterile 0.9% saline, just before the experiment. Streptozotocin was administered into the lateral ventricle and metformin, N-acetyl cysteine, and saline were injected intraperitoneally.

Infusion and surgical procedures

All surgical procedures were performed by an anesthetic effect of ketamine/xylazine. ketamine-xylazine (50 mg/kg ketamine-5 mg/kg xylazine). Stereotaxic coordinates for lateral ventricle were incisor bar (-3.3 mm), 0.8mm posterior to the bregma, ±1.4 mm lateral to the sagittal suture and 3.4 mm from the top of the skull (Paxinos and Watson, 1998). Each injection unit was injected by polyethylene tubing to 25µl Hamilton syringe. The left and right ventricles were infused with a 10µl solution on each side (20µl/rat) for about 3 minutes.

Inhibitory Avoidance Apparatus (Shuttle box)

Inhibitory Avoidance Apparatus accounted for two rooms of the similar size (20 × 20 × 30 cm). There was also a guillotine door between two rooms separating chambers. The walls and floor of one compartment consisted of white opaque resin and another one was dark. Intermittent electric shocks (50 Hz, 3 seconds, 1.5mA intensity) were delivered to the grid floor of the dark compartment by an isolated stimulator. Each animal was gently placed in the white compartment and after 5 seconds the guillotine door was opened and the animal was allowed to enter the dark module (Rafiq, 2013).

Once the animal entered with all four paws to the next chamber, the guillotine door was closed and the rat was immediately withdrawn from the compartment (this trial was repeated after 2 minutes). As in the acquisition trial, when the animal entered the dark (shock) compartment the door was closed, and a foot shock (50 Hz, 1.5 mA, 3 seconds) was immediately delivered to the grid floor of the dark room. After 20 seconds, the rat was removed from the apparatus and placed temporarily into its home cage. Two minutes later, the animal was retested in the same way as in the previous trials; if the rat did not enter the dark compartment during 300 sec, a successful acquisition of inhibitory avoidance response was recorded. Otherwise, when the rat entered the dark compartment (before 300 seconds) a second time, the door was closed and the animal received the shock again. Twenty-four hours later, each rat was again placed in the light chamber 5 seconds the door was opened and the latency with which the animal entered the dark chamber (STL) and the total time spent in dark compartment (TDC) was recorded in the absence of electric foot shocks, as an indicator of inhibitory avoidance behavior (Darbandi, 2016 a).

Tissue preparation and histological stain

All rats were anesthetized with 3.5% chloral hydrate (35 mg/100 g, i.p.) and perfused through the left ventricle with phosphate buffer (PBS, 0.1 M, pH 7.4), followed by 4% paraformaldehyde (PF) in pre-cooled physiological saline. The brains were removed for the post-fixed process by PF (24 or 48 hours) and were then processing and embedding in paraffin. Coronal sections, 10µm in thickness, had been taken from the dorsal hippocampus and stained with hematoxylin and eosin (H&E). Finally, the numbers of damaged and intact neurons in the hippocampal CA1 pyramidal layer were specified (Darbandi, 2016 a). using a light microscope (BX40, Olympus, New York, USA) connected to a camera (Olympus, DP12), and quantitatively analyzed and counted by Image J software (Neuroscience Research Center of Shahid Beheshti University, Iran).

Biochemical analysis

Biochemically, to analyze the level of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and Ferric Reducing Ability of Plasma (FRAP), blood samples from the right atrium of the heart were collected. MDA, as a product of lipid per oxidation originating from active oxygen radicals, was used as a biomarker for oxidative damage. Lipid peroxidation was assessed indirectly by measuring the secondary products, such as MDA via the thiobarbituric acid method. The assay was based on the reaction of MDA with thiobarbituric acid (TBA) (Pintana, 2012). Enzyme super oxide dismutase (SOD) rapidly dismutase sunivalently reduced oxygen $O_2.^-$, the superoxide anion radical ($2O_2.^- + 2\ H^+ + O_2 + H_2O_2$). It has been proven that enzyme is presumably useful for studying the participation of the radical in reactions involving oxygen such as autoxidations. The inhibition of pyrogallol autoxidation brought about by superoxide dismutase can be employed in a rapid and convenient method for the determination of the enzyme (Marklund, 1974). Catalase activity was measured based on the method of Aebi (Aebi, 1984) In the ultraviolet range, H_20_2 shows a continual increase in absorption with decreasing wavelength. The decomposition of H_20_2 can be followed directly by the decrease in absorbance at 240 nm. The difference in absorbance per unit time is a measure of the catalase activity. FRAP assay depends upon the ferric tripyridyltriazine (Fe(III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe(II)-TPTZ) by a reductant at low pH. Fe(II)-TPTZ has an intensive blue color and can be monitored at 593 nm (Gohari, 2011) FRAP method is sensitive to the measurement of the total antioxidant power of the fresh biological fluids. The results were expressed as nmol/ml, U/ml, and mmol/l.

Verification of cannulas placements

At the end of the trial, in order to assess the accuracy of coordinates of the surgical site and the injection, 1 microliter of methylene blue 1% (w/v) solution was bilaterally injected. Animals' brain was removed and fixed in 10% formaldehyde over 10 days. Sections were examined to determine the location of the cannulas aimed at the lateral ventricle. The cannulas placements were verified using the atlas of Paxinos and Watson (paxinos and watson., 1998).

STATISTICAL ANALYSIS

The avoidance test and biochemical data are expressed as means± S.E.M. The statistical analyses were performed using one-way analysis of variance (ANOVA). The post-hoc comparison of means was carried out with the Tukey test for multiple comparisons, when appropriate. Statistical results were analyzed using SPSS 16.0 software. Differences between groups in the average number of intact and damaged neurons in the hippocampal CA1pyramidal layer were analyzed using Graph Pad Prism Software (version 5.00). A value of $P<0.05$ was considered statistically significant.

Table 1: Comparison of serum analysis in the experimental groups

Index measured groups	MDA (nmol/ml)	FRAP (mmol/l)	SOD (U/ml)	(ml/U)CAT
Control	2.30±0.605 [a]	0.8218±0.062 [a]	10.37±1.125 [a]	2.80±0.12 [a]
STZ	5.14±0.255 [b]	0.338±0.019 [b]	3.17±0.375 [b]	2.28±0.04 [b]
STZ + Met (1week)	2.1±0.16 [a]	0.76 ±0.09 [a]	8.22±0.565 [a]	2.84± 0.18 [a]
STZ + Met (3weeks)	2.58±0.34 [a]	0.72±0.11 [a]	7.87±0.42 [a]	2.71±0.25 [a]
STZ + Met (11weeks)	5.33 ±0.63 [b]	0.3325± 0.58 [b]	2.70±1 [b]	2.23±0.09 [b]
STZ + Met (11weeks)+NAC (11weeks)	2.74±0.594 [a]	0.6675±0.053 [a]	6.335±0.208 [a]	2.79±0.06 [a]
NAC (11weeks)	2.02±0.526	0.8963±0.015	9.89±0.96	2.98±0.26

The values with different superscript letters within the same column significantly differ at P<0.001. Malondialdehyde (MDA); Ferric Reducing Ability of Plasma (FRAP); superoxide dismotase (SOD); catalase.

RESULTS

The comparison of memory retrieval between groups

As fig. 1 shows, intraventricular injections of streptozotocin (3mg/kg) did not show any delay time in entering to the dark room compared to the control group which means a significant reduction in learning and memory retention over 24 hours. One-way ANOVA showed an improvement in the STZ + metformin group (one and three weeks' infusion) meaning that metformin can ameliorate memory retention.

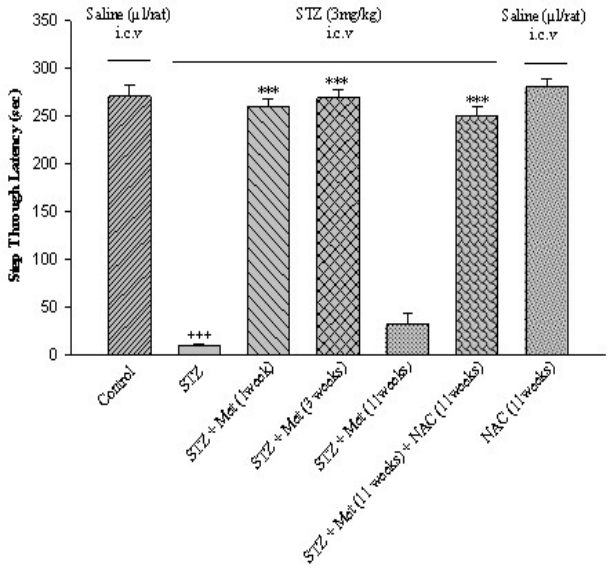

Fig. 1: Streptozotocin induced amnesia in rats with

Alzheimer's disease compared to the control group. Injections of metformin (200mg/kg, i.p.) for one week and three weeks but not for eleven weeks significantly improved memory and delay in entering the dark room increased. The administrations of NAC in combination with metformin significantly improved memory retention compared to the STZ group. In the N-acetyl cysteine group no significant change was observed as compared with the control group. Results are expressed as (Mean ± SEM) for all the eight animals. $^{+++}$P <0.001 is compared with the control group. ***P<0.001 is compared to the STZ group.

Tukey test revealed that treatment with metformin for one or three weeks significantly showed the delay time in entering to the dark room, suggesting the improvement of the memory. In the group of STZ + metformin (eleven weeks) the administration of metformin did not elevate delay time in moving to the dark chamber. Accordingly, treating with metformin over eleven weeks did not represent any significant effect on memory retention; However, In the group of STZ+ metformin +N-acetyl cysteine, administrations of drug combinations of metformin and N-acetyl cysteine for eleven weeks remarkably improved memory retention compared to the STZ group [F (6,52) = 47.131, P<0.001]. There were no significant differences between two groups of N-acetyl cysteine and control in the delay time in entering the dark room.

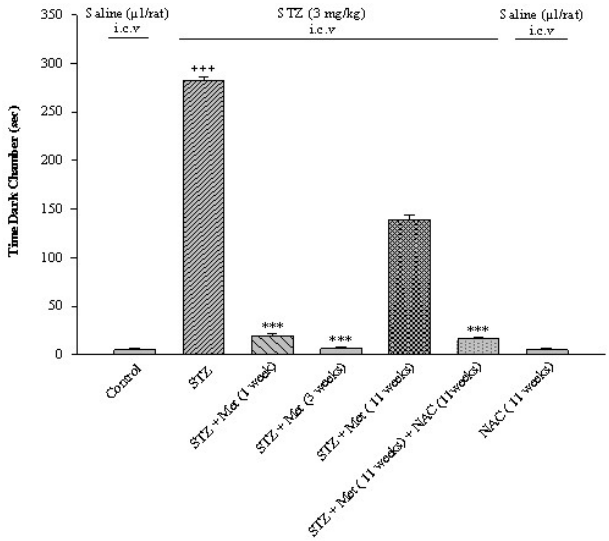

Fig. 2: The time spent in the dark room in rats with Alzheimer's disease increased compared to the control group. Injections of metformin for one week and three weeks but not for eleven weeks in rats with Alzheimer's disease decreased this time and improved memory. The administrations of NAC in combination with metformin significantly decreased this time compared to the STZ group. In the N-acetyl cysteine group no significant change was observed as compared with the control group.

Results are expressed as (Mean ± SEM) for all the eight animals. $^{+++}$P <0.001 is compared with the control group. ***P <0.001 is compared to the STZ group.

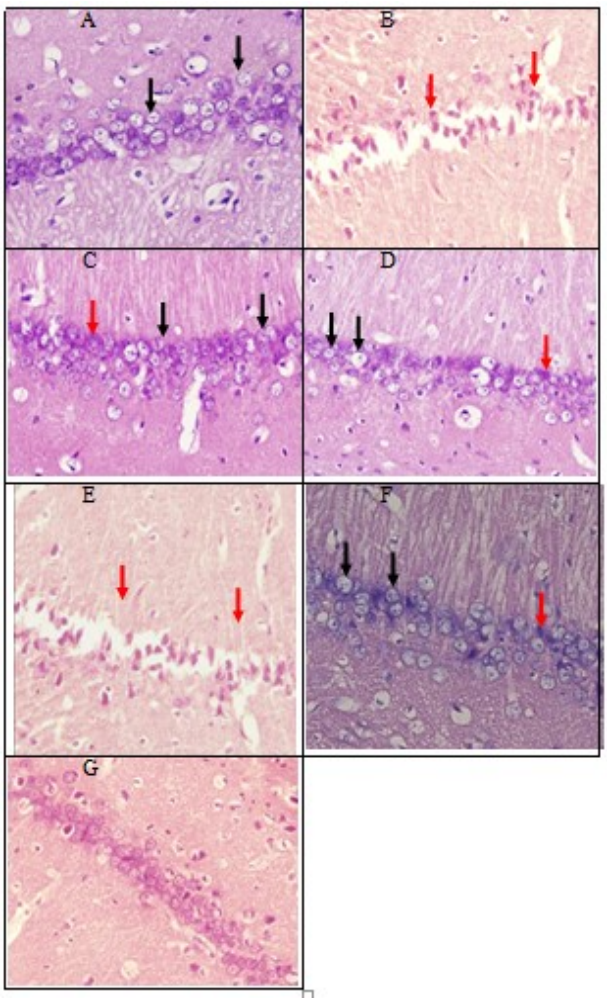

Fig. 3: Photomicrographs of typical coronal sections through the CA1 pyramidal neurons of the hippocampus showing Hematoxilin & Eosin in A: Control, B: STZ group, C: STZ+ Met (one week), D: STZ+ Met (three weeks), E: STZ + Met (eleven weeks) and F: STZ+ Met + NAC (both eleven weeks),G: NAC (eleven weeks), Black arrows show intact cells and red arrows show degenerating pyramidal cells ×400

Results of the one-way ANOVA indicated that the time spent on the darkroom was significantly different among the mentioned groups. During the retention test, after 24hthe STZ group had an increased TDC, compared to the control group. The STZ + metformin groups (one and three weeks) had a decreased TDC, compared to the STZ group. While in the group of STZ + metformin (eleven weeks) this time increased compared to the STZ group. Treatment of animals In this group (STZ + metformin +N-acetyl cysteine) with N-acetyl cysteine significantly decreased TDC, compared to the STZ group [F (6, 52)= 110.265, P<0.001]. In the N-acetyl cysteine group was not any significant difference in the time spent on the darkroom compared with the control group (fig. 2).

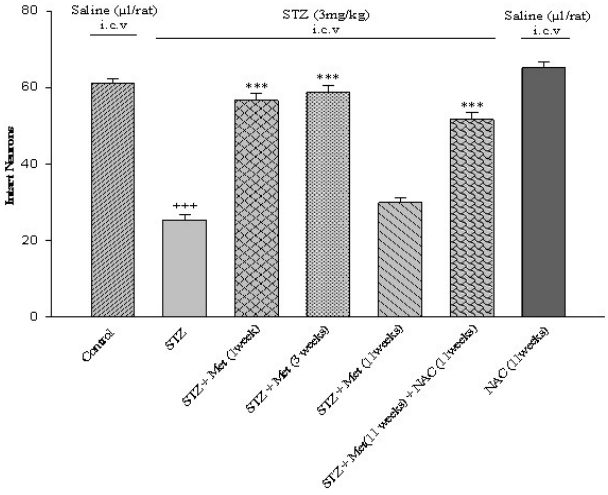

Fig. 4: Neurons density in the CA1 area of the hippocampus in the brains of all groups. Results are expressed as (Mean ± SEM). $^{+++}$P<0.001 is compared with the control group. ***P<0.001 is compared to the STZ group.

Histology and comparison of the average number of intact neurons

After performing the behavioral tests, in order to consider more morphological changes in rats' hippocampus, histological examination was carried out with H&E staining. The H&E-stained samples did not show any substantial neuronal damages in the control, while the infusion of STZ (3mg/kg) produced a significant neuronal death in hippocampal CA1 neurons in the STZ. Treating with metformin for one and three weeks could protect neurons from STZ -induced damage. In the group of STZ+ metformin +N-acetyl cysteine the administrations of both N-acetyl cysteinein combination with metformin positively protected neurons from STZ -induced damage. Also, the N-acetyl cysteine group did not show any substantial neuronal damages (fig. 3).

To survey the number of neurons existing in the hippocampal CA1 area, we observed a considerable reduction (P<0.001) in STZ group in comparison with control group. Examination of the present data also showed that in the groups of STZ+ metformin (one and three weeks) the number of intact neurons in the pinpointed area significantly increased compared to the STZ group. In the group of STZ+ metformin (eleven weeks) intact neurons in the CA1areadid not manifest any significant difference compared to the STZ group (the number of intact neurons is similar in these two groups). But in the group of STZ + metformin + N-acetyl cysteine, co-administration of metformin and N-acetyl cysteine (both eleven weeks) significantly increased the number of intact cells. There were not any significant differences

between N-acetyl cysteine and control groups in the number of intact neurons [F (6, 52) = 106.554, P<0.001], (fig. 4).

The results of the biochemical analysis

As it can be seen in table 1 the plasma malondialdehyde (MDA) levels estimated in STZ and STZ+ metformin (eleven weeks) groups were significantly high in comparison with the control group [F(6,24)=20.621, P<0.001].This indicates lipid per oxidation is significantly increased in these groups. Besides, the activities of super oxide dismutase (SOD), catalase (CAT) and Ferric Reducing Ability of Plasma (FRAP) significantly decreased in these groups compared to the control group [F (6, 24) =30.375, P<0.001], [F (6, 24) =22.848, P<0.001] and [F (6, 24) =25.015, P<0.001] respectively. Administration of both N-acetyl cysteine and metformin (eleven weeks) significantly decreased the level of MDA and increased the activity of superoxide dismutase(SOD), catalase(CAT) and Ferric Reducing Ability of Plasma(FRAP) compared to the STZ group (P<0.001). In the groups of STZ+ metformin (one and three weeks) and N-acetyl cysteine, the levels of MDA, SOD, CAT and FRAP showed no significant differences compared to the control group (P>0.05).

DISCUSSION

According to the results achieved from these examinations, the ICV injections of STZ (3mg/kg, 10 µl in each ventricle) on the first and third days after surgery significantly reduced memory retention (fig. 1, 2), intact pyramidal cells (fig. 4) and serum levels of oxidative stress biomarkers (tab. 1) compared to the control group. Histological study revealed that CA1 neurons in pyramidal layer in the control (fig. 3A) have natural morphological fig containing large, circular and regular; they seem to be very clear and had a single nucleus which is located in the same layer; However, in STZ group (fig. 3B), the neurons had become smaller with surrounding huge free spaces and the number of dead cells increasingly was becoming more and more. In this group, the pyramidal layer did not show a unified and regulated structure and remarkable changes in the morphology, such as shrinkage and atrophy was found.

Previous studies shed light on our findings (Darbandi, 2016 a; Ramezani, 2016; Darbandi, 2016 b). ICV injections of STZ in rats causes progressive long-term deficits on learning and cognitive performance which is similar to sporadic Alzheimer's disease (Mehla, 2013; Rai, 2013; Esmaeili, 2018). This leads to cell death via three major pathways: DNA methylation, nitric oxide production and free radical generation (Eleazu, 2013). Besides, Protein aggregation, mitochondrial dysfunction, and glutamate excite toxicity are other key factors that collectively contribute to the neuronal death. It has been manifested by growing documents that administration of STZ generates free radicals resulting in increased oxide-nitrosative stress in rodents (Kamat, 2015). Oxidative stress is usually associated with inflammation in the brain cells such as microglia and astrocytes which produce various pro inflammatory mediators, neurotoxic factors and free radicals (Asadbegia, 2016). There is evidence that STZ injections into the brain induce insulin resistance by damaging IR signaling and lead to increase processed amyloid precursor protein to beta-amyloid and tau pathology. Tau protein and beta-amyloid are responsible for upcoming pathological events that result in a lack of cognitive performance, neuropsychiatric alterations, and eventually lead to neuronal death (Chung, 2015).

In this study, treatment of Alzheimeric rats with metformin (200 mg/kg i.p.) for one week and three weeks significantly improved the memory retention (fig. 1, 2). The number of intact neurons in the CA1 pyramidal layer increased and the number of dead cells decreased (fig. 3C, D). Treatment with metformin in these groups neutralized STZ induced toxicity and allowed the proliferation of neurons in the hippocampus. In these groups, serum oxidative stress biomarkers improved compared to the STZ group (tab. 1).

Many previous studies confirm our findings (Zhao. 2014; Zhao, 2014). Metformin is an anti hyperglycemic factor used for the management of type 2 diabetes. It leads to reduce insulin resistance by its positive effects on insulin receptor expression and tyrosine kinase activity (Viollet, 2012). Alzheimer's disease has been considered as type 3diabetes and characterized by impaired insulin actions and neuronal insulin resistance leading to excessive generation and accumulation of beta-amyloid (Rojas, 2013). Metformin can rapidly pass via blood-brain barrier after oral administration and it has several beneficial effects on the brain such as antioxidant, anti-inflammatory properties and neuroprotective effects (El-Mir, 2008; Mousavi, 2018; Markowicz, 2017). Metformin reduces reactive oxygen species (ROS), increases the antioxidant enzyme activities, decreases the biomarkers of lipid peroxidation and decreases advanced glycosylation and produces directly through an insulin-dependent mechanism and indirectly through reduction of hyperglycemia (Rojas, 2013; Memisogullari, 2008). Metformin inhibits the mitochondrial permeability transitional pore (MPTP) as well as decreases the inflammatory cytokines secretion (Mahrouf, 2006). It seems that *in vivo* metformin would certainly exert its antioxidant activity by decreasing the markers of lipid peroxidation (Pavlovic, 2000), increasing the antioxidant enzyme activities, and inhibiting the formation of AGEs (Markowicz, 2017). Previous studies also demonstrated that metformin could regulate lipid and glucose metabolism via activation of AMP-activated protein kinase (AMPK) which acts as a modulator of long-term potentiation and it is necessary for the memory formation (Pintana, 2012).

Also in this study administration of STZ+ metformin (eleven weeks) did not produce any considerable difference on memory retrieval (fig. 1, 2), the number of intact cells in the CA1 pyramidal layer (fig. 3E) and serum oxidative stress markers (tab. 1) compared with the STZ group.

Although it has been approved that metformin would be beneficial for the treatment of Diabetes Mellitus type-2, there is a fear associated with the potential danger of metformin-induced lactic acidosis which is still subject to debate (Esteghamati, 2013). One basic kind of lactic acidosis results from the accumulation of lactate via glycolysis in the absence of oxygen. Another kind of lactic acidosis, MALA (metformin-associated lactic acidosis), appears during isolation of hepatocytes. Metformin inhibits mitochondrial respiratory chain complex Iin a concentration-dependent manner and impairs gluconeogenesis (Yilmaz, 2005). An obvious increase in plasma lactate concentration observed over metformin exposure in vivo which was correlated with the inhibition of mitochondrial oxidative phosphorylation *in vitro* (Yilmaz, 2005). Although in some studies it has been demonstrated that metformin reduces oxidative stress (Rojas, 2013; Cheraghi, 2014; Memisogullari, 2008), others reveal that metformin increases oxidative stress in diabetic patients (Khouri, 2004; Pavlovic, 2000). It was showed that metformin may raise oxidative stress by increasing homocysteine levels which is associated with the generation of superoxide anions more and more and decreased activity of antioxidant enzymes (Pavlovic, 2000). In the previous results obtained showed that metformin in an in vitro model is not a very good scavenger of reactive oxygen species (Khouri, 2004). However, recently many studies have shown the strong correlation of long-term metformin use with biochemical vitamin B12 deficiency and anemia. Vitamin B12 plays a vital role to play in red blood cell formation, the metabolism of homocysteine and in nerve cell physiology (Thomakos, 2017).

Although in the N-acetyl cysteine group, compared to the control group, no significant change was illustrated, but in the group of STZ+ metformin (eleven weeks) + N-acetyl cysteine (eleven weeks), co-dministration of metformin and N-acetyl cysteine improved memory retrieval (fig. 1, 2), increased the number of intact neurons (fig. 4) and serum oxidative stress biomarkers (tab. 1) compared to the STZ group.

It is vividly known that NAC stimulates the cytosolic enzymes involving in glutathione regeneration in which it can act by direct reactions between reducing thiol group and reactive oxygen species. NAC, as a potent antioxidant, can attenuate 3-4, methylene dioxy methamphetamine (MDMA). It has been demonstrated that NAC can prevent programmed cell death in cultured neuronal cells. It can also increase mitochondrial complex activity in Both in Vitro and in Vivo Models. Moreover, NAC can stimulate GSH synthesis, promote detoxification, enhance Glutathione-S-transferase activity and directly act as reactive oxidant radicals (De Flora, 2001). Besides, being active as a GSH precursor via enhancing intracellular concentrations, it can create a protective effect against cytotoxicity (De Flora, 2001). In an in vitro model that disrupts mitochondrial electron transport function, NAC can prevent oxidative damage and cell death. Another study showed that in Parkinson disease, NAC is a potent scavenger of both H2O2 and toxic quinines which are derived from dopamine contributing to cell death. It can also prevent dopamine-mediated inhibition of Na^+, K^+-ATPase in rat brain (Bagh, 2008). It has been illustrated that NAC increases glutathione levels in the mouse brain, increase mitochondrial Complex I activity in nerve cells, reduce biomarkers of Oxidative-stress-induced damages, and protect against dopamine cell death from mitochondrial permeability transitional pore toxicity (Sharma, 2007). NAC, as an antioxidant, inhibits the transcription factors activity of NF-kB and AP-1, it can also influence the signaling pathway of MAP-kinase which results in a reduction in the oxidative stress (Lappas, 2003).

Despite the fact that metformin (as insulin sensitizers) and N-acetyl cysteine (as an antioxidant) have different actions, and researchers have shown that there seem to be no beneficial effects of these drug combinations during the ovulation in the process of intra cytoplasmic sperm injection (ICSI) (Cheraghi, 2014), in our work, we proved that not only administration of both acute and chronic metformin have different effects on memory retrieval, hippocampal CA1 neurons, and level of oxidative stress factors but also administration of drug combination of N-acetyl cysteine and metformin can improve the memory retrieval and some factors of serum oxidative stress in streptozotocin-induced Alzheimer rats.

CONCLUSION

Acute administration of metformin improved memory retrieval, hippocampal CA1 neurons, and level of oxidative stress factors in streptozotocin-induced Alzheimer's rats. Chronic administration of metformin did not have a positive effect on the above mentioned factors. However, Co- administration of N-acetyl cysteine with Chronic metformin improved these factors. Although more research is needed but it seems the chronic use of metformin for example in diabetic patients should be more cautious.

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