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Effect of Letrozole on hippocampal Let-7 microRNAs and their correlation with working memory and phosphorylated Tau protein in an Alzheimer's disease-like rat model

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Abstract

Background: Let-7 microRNAs (miRNAs) may contribute to neurodegeneration, including Alzheimer's disease (AD), but, they were not investigated in Streptozotocin (STZ)-induced AD. Letrozole increases the expression of Let-7 in cell lines, with conflicting evidence regarding its effects on memory. This study examined Let-7 miRNAs in STZ-induced AD, their correlation with memory and hyperphosphorylated Tau (p-Tau) and the effects of Letrozole on them.

Methods: Seven groups of adult Sprague Dawley rats were used: Negative control, Letrozole, Letrozole Vehicle, STZ (with AD induced by intracerebroventricular injection of STZ in artificial cerebrospinal fluid (aCSF)), CSF Control, STZ + Letrozole (STZ-L), and CSF + Letrozole Vehicle. Alternation percentage in T-maze was used as a measure of working memory. Let-7a, b and e and p-Tau levels in the hippocampus were estimated using quantitative real-time reverse transcription–polymerase chain reaction (qRT–PCR) and enzyme-linked immunosorbent assay (ELISA), respectively.

Results: Significant decreases in alternation percentage and increase in p-Tau concentration were found in the STZ, Letrozole and STZ-L groups. Expression levels of all studied microRNAs were significantly elevated in the Letrozole and the STZ-L groups, with no difference between the two, suggesting that this elevation might be linked to Letrozole administration. Negative correlations were found between alternation percentage and the levels of all studied microRNAs, while positive ones were found between p-Tau concentration and the levels of studied microRNAs.

Conclusions: This study shows changes in the expression of Let-7a, b and e miRNAs in association with Letrozole administration, and correlations between the expression of the studied Let-7 miRNAs and both the status of working memory and the hippocampal p-Tau levels. These findings might support the theory suggesting that Letrozole aggravates pre-existing lesions. They also add to the possibility of Let-7's neurotoxicity.

Keywords: Letrozole, Let-7 microRNAs, Alzheimer's disease, Tau, Memory

Introduction

Alzheimer's disease (AD) is a neurodegenerative condition which causes aging-related cognitive impairment and dementia [1]. It accounts for 50% to 75% of all dementia cases, making it the chief cause of dementia [2]. It is pathologically characterized by accumulation of beta-amyloid (Aβ) plaques, and fibrillary tangles of aggregated hyperphosphorylated Tau protein [3].

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The role of microRNAs (miRNAs) in numerous diseases is the focus of a lot of recent studies. miRNAs are approximately 22-nucleotide long, non-coding RNAs that function as gene expression regulators at the post transcriptional level. The Let-7 miRNAs are conserved in multiple species, and are abundantly expressed in the brain [4, 5]. Let-7 miRNAs play roles in cellular differentiation, tumor suppression and neurodegeneration/neurotoxicity [6–8]. Various members of the Let-7 family were found to be differentially expressed in the cerebrospinal fluid of AD patients [5].

Up to our current knowledge, levels of Let-7 miRNAs were not previously investigated in a Streptozotocin (STZ)-induced AD model, neither was the effect of Let-7 modulation on the memory of the animals in such model.

Multiple drugs were found to increase the expression of Let-7 in various cell lines [9, 10], an example of which is Letrozole [11, 12], which is a potent aromatase inhibitors with experimental evidence documenting its high permeability across the blood brain barrier (BBB) [13].

Memory changes are a somewhat common complaint in patients treated with Letrozole. However, there are several conflicting studies examining the possible effects of aromatase inhibitors on memory. Some studies have shown that Letrozole administration resulted in memory impairment [14, 15], while others have shown that Letrozole improved the acquisition of working memory and that inhibition of brain estrogen synthesis may have beneficial effects on spatial memory [16, 17].

Therefore, this study was designed to examine Let-7 miRNAs levels in the hippocampi of STZ-induced AD-like state rats, and whether they can be correlated with memory status and/or phosphorylated Tau levels. It also aims to assess the potential effects of Letrozole on the memory and levels of Let-7 miRNAs in normal animals as well as animals with an existing STZ-induced AD-like state. This can possibly provide an insight into a mechanism by which Letrozole could exert an effect on cognition via microRNA modulation.

Methods

Animals and grouping

A total number of 51 adult Sprague Dawley rats of the same age, weighing 150–200 gm., were subjects of this study. Rats were supplied by the animal unit of the Medical Experimental Research Center (MERC), faculty of Medicine, Mansoura University. An ethical approval for this study was obtained from the Institutional Animal Care and Use Committee (IACUC), Zagazig University, in line with the ARRIVE guideline.

Rats were housed in plastic rodent cages (3–5 per cage) under hygienic and environmentally controlled conditions i.e., ambient temperature of 25 ± 2 °C, and a cycle of 12-h light/ 12-h dark. They were allowed 14 days for acclimatization, during which, and throughout the entire period of the study, they were fed standard commercial rat chow and had free access to food and water.

Following the acclimatization, rats were randomly divided into seven groups as follows (Fig. 1):

Group 1: Negative control Control Group ($n = 6$): No procedure or treatment was applied to this group. It served to establish basal levels of studied parameters (Cognitive function, hippocampal phosphorylated Tau level and Let-7a, b, e microRNA levels).

Group 2: Letrozole Group ($n = 9$): Letrozole (Synthon, Netherlands) was given at a daily dose of 2.5 mg/kg [16] by oral gavage [17]. Letrozole was dissolved in 0.5% carboxy methylcellulose (CMC) solution, and was given for 15 days [15].

Group 3: Letrozole Vehicle (LV) Control Group ($n = 6$): Rats were given a solution of 0.5% CMC daily, by oral gavage, for 15 days.

Group 4: STZ Group ($n = 9$): In this group, sporadic Alzheimer's disease-like state was induced using intracerebroventricular injection (ICV) of 3 mg/kg single dose of Streptozotocin (Sigma-Aldrich, USA) dissolved in artificial cerebrospinal fluid (aCSF).

Group 5: CSF Control Group ($n = 6$): Rats were intracerebroventricularly injected with aCSF only.

Group 6: STZ + Letrozole (STZ-L) Group ($n = 9$): Sporadic Alzheimer's disease-like state was induced using ICV injections of 3 mg/kg of Streptozotocin dissolved in aCSF, as mentioned in group 4. Following the surgery, rats were allowed a recovery period of 7 days then, given 2.5 mg/kg Letrozole by oral gavage, for 15 days.

Group 7: CSF + Letrozole Vehicle (CSF-LV) Control Group ($n = 6$): Rats were administrated aCSF via ICV injections, allowed to recover for 7 days after surgery, then given 0.5% CMC daily, by oral gavage, for 15 days.

Induction of AD by ICV injection of STZ

Animals were anesthetized and the general guidelines and steps for stereotaxic surgery were followed as previously mentioned [18]. A single dose of Streptozotocin (3 mg/kg [19]), in 10 µL aCSF (119 mM NaCl, 26.2 mM $NaHCO_3$, 2.5 mM KCl, 1 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 10 mM glucose and 2.5 mM $CaCl_2$ in distilled water, then passed through an 0.22 µm filter for sterilization [20]) was injected into the two lateral ventricles using coordinates according to the rat stereotaxic brain atlas [21], and the Waxholm Rat atlas via the scalable brain atlas [22–25]: anteroposterior from bregma (AP) = − 0.9 mm, mediolateral from the midline (ML) = ± 1.5 mm and dorsoventral from the skull (DV) = 3.6 mm. Animals were allowed a week to fully recover prior to administration of any further treatment. Typically, the effects of

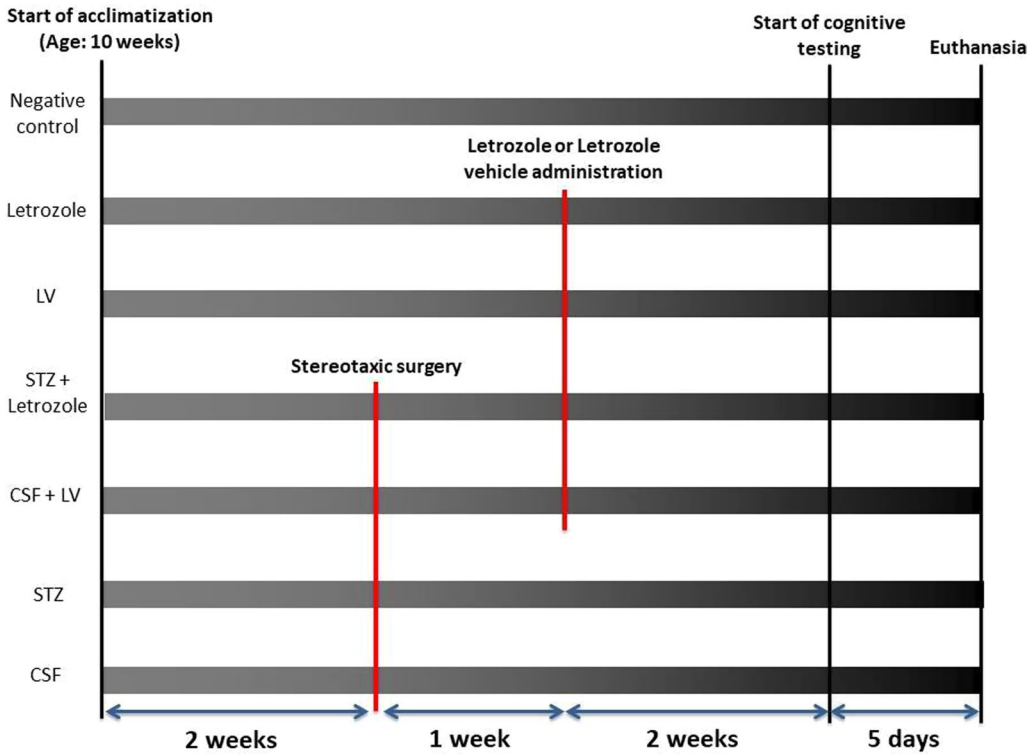

Fig. 1 Timeline of animal experiments. Briefly, following a 2-week acclimatization period, animals were randomly divided into seven groups: Negative control, Letrozole, Letrozole Vehicle (LV), STZ, CSF Control, STZ + Letrozole, and CSF + Letrozole Vehicle (CSF + LV) Control. The experimental time frame was 26 days from the experimental starting point, at which the stereotaxic surgery was performed on the STZ, CSF Control, STZ + Letrozole, and CSF + LV groups, to the animal euthanasia point, at which hippocampal samples were collected

STZ on memory functions start to manifest 15 days following the injection [19]. Hematoxylin and eosin (H&E) staining of hippocampal sections from representative samples was done to further confirm the presence of neurodegeneration.

Testing of working memory: spontaneous alternation in a modified T-maze

A manually-run T-maze with a central partition and a guillotine door at each goal arm was constructed. The maze design was adapted from Deacon and Rawlins, (2006) and their protocol was followed. In brief: No habituation to the maze was allowed, as it is the novelty of the maze that motivates the spontaneous exploration/alternation. For each rat, one sample trial and five choice runs were performed per day for two days, amounting to a total of 12 trials per rat, and a total of ten possible alternations. In the sample run, each rat was placed in the starting area at the bottom of the T and allowed to select a goal arm. The rat was kept for 30 s in the selected arm by quietly sliding down the guillotine door, and then it was removed and put back in its cage for an inter-trial interval of 10 min. In the choice runs, the central partition was removed, and the guillotine door of the arm chosen in the sample run was raised again. The rat was placed again at the starting point, facing away from the goal arms and allowed to pick one of the two open goal arms. Each trial took 1–2 min. A rat's choice of the opposite arm to the one chosen in the preceding trial was defined as an alternation (a correct choice). A percentage or proportion correct choice (alternation) per animal was calculated as follows [26, 27]:

$$\frac{Numberofalternations(Correctchoices)}{Totalpossiblealternations} \times 100$$

Biochemical and molecular testing

Animals were euthanized by cervical dislocation following The University of Texas at Austin [28] guideline and the brain was immediately removed from the skull. The hippocampi were rapidly dissected following the method described by Spijker [29] and hippocampal samples were submerged in either RNAlater RNA stabilization reagent (Qiagen, USA, Cat No./ID: 76104), or sterile

Phosphate-buffered saline (PBS) and stored at a temperature of − 80 °C for biochemical analysis [30].

Total RNA, including microRNAs, was extracted from the hippocampus using the miRNeasy Mini Kit (Qiagen, USA, Cat No./ID: 217004) following the kit's manual, and the purity and concentration of the extracted total RNA were spectrophotometrically determined using a NanoDrop [31]. Reverse transcription (RT) reaction was done for each sample using the TaqMan™ microRNA reverse transcription kit (Applied biosystems, USA, Cat no.: 4366596). A specific TaqMan microRNA assay was used, following the manufacturer's instructions, in qRT–PCR to detect each of the three target microRNAs (Let7a, b and e) as well as U6 small nuclear RNA as an endogenous control (rno-let-7a-5p [Assay ID: 000377], rno-let-7b-5p [Assay ID: 000378], rno-let-7e-5p [Assay ID: 002406] and U6 snRNA [Assay ID: 001973], Applied biosystems, USA, Cat no.: 4427975). Changes of miRNA expression were calculated for each of the three tested miRNAs using the 2^(−ΔΔCt) method [32, 33].

Rat p-Tau (Ser396) protein ELISA kit (Bioassay technology laboratory, Shanghai, China) was used according to the manufacturer's instructions to measure p-Tau concentration in the hippocampus.

Statistical analysis

The statistical analysis was done using IBM SPSS Statistics for Windows, version 22 (IBM Corp., Armonk, N.Y., USA). For quantitative variables, mean and standard deviation were computed. Median and range were computed in case of data that were not normally distributed (non-parametric data). One way analysis of variance (ANOVA) test was used for comparison of means of more than two groups in normally distributed data, and Kruskal–Wallis test was used in case of data that were not normally distributed. Spearman's correlation coefficient was used as a measure of the strength of association between two variables. For all the mentioned statistical tests, P values of >0.05 indicated non-significant results, while P values of <0.05 indicated significant results.

Results

Verification of neurodegeneration in the STZ-injected rats via histological examination

Histological examination of H&E stained hippocampal sections from representative STZ-injected rats showed signs of degeneration as well as inflammatory changes (Fig. 2).

Comparing the state of working memory (in alternation percentage) and p-Tau levels among the different studied groups

There were statistically significant differences between different groups in both alternation percentage and p-Tau concentration (Table 1). The STZ, Letrozole and STZ + Letrozole groups showed significant reductions in alternation percentage compared to all control groups ($P<0.001$). These three groups also showed significant differences in alternation percentages compared to each other ($P<0.001$), with the STZ + Letrozole group showing the most reduction in alternation percentage (Mean ± SD 17.78 ± 4.41), followed by the

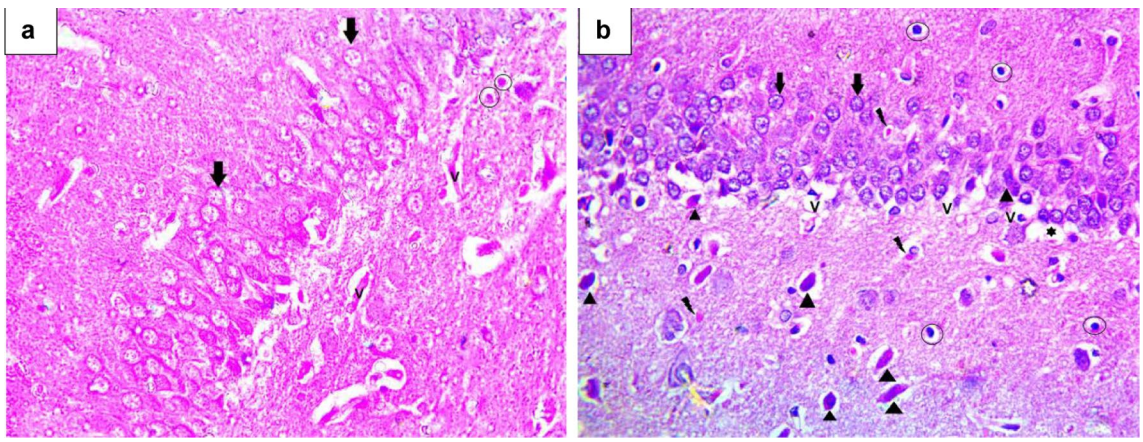

Fig. 2 Sections in the hippocampus for confirmation of neurodegeneration in the STZ animals. **a** Section from normal hippocampus showing normal thickness of the pyramidal layer with rounded granular cells (arrows). Astrocytes (circles) and blood vessels (V) are found in the molecular layer (Hematoxylin and Eosin ×400) **b** Section from STZ-induced AD hippocampus with CA2 showing some degenerated pyramidal cells with neurofibrillary tangles (arrowheads) within the normal pyramidal cells (arrows), cell loss (asterisks) and other vacuolated cells (V) in the basal layer of pyramidal cells. Enlarged and excess astrocytes (circles) are found in the molecular layer (downward) and the polymorphic layer (upward). Depositions of amyloid protein (broken arrows) are found in the molecular layer (Hematoxylin and Eosin ×400)

Table 1 Alternation percentage and p-Tau concentration among the studied groups

Groups	Parameter	
	Memory status (Alternation percentage) Mean ± SD Range	**p-Tau concentration (pg/ml) Mean ± SD Range**
Negative control ($n=6$)	76.67 ± 12.12^{a} 60–90	16.87 ± 2.77^{a} 13.6–19.32
CSF ($n=6$)	81.67 ± 14.72^{a} 60–100	18.29 ± 2.77^{a} 14.78–21.14
LV ($n=6$)	73.33 ± 8.16^{a} 60–80	17.70 ± 2.05^{a} 13.74–19.2
CSF-LV ($n=6$)	78.33 ± 11.69^{a} 60–90	18.89 ± 0.80^{a} 18–20.05
STZ ($n=9$)	35.56 ± 8.82^{b} 20–50	22.36 ± 0.83^{b} 21.14–23.61
Letrozole ($n=9$)	53.33 ± 8.66^{c} 40–60	23.24 ± 1.23^{b} 21.1–24.53
STZ-L ($n=9$)	17.78 ± 4.41^{d} 10–20	23.37 ± 1.46^{b} 21.66–25.2
F	49.21	18.55
P	< 0.001**	< 0.001**

F: ANOVA test, **Highly significant ($P<0.01$)

Groups with different letters have statistically significant ($P<0.05$) differences

STZ group (35.56 ± 8.82) then the Letrozole group (53.33 ± 8.66). The STZ, Letrozole and STZ + Letrozole groups also showed significant elevations in p-Tau levels compared to the control groups ($P \leq 0.001$).

Comparing Let-7 miRNAs expression (in fold change) among the studied groups

Tables 2, 3, 4 and 5 show that there were statistically significant differences between the different groups in the expression of Let-7 a, b and e. Using the least significant difference (LSD) test to compare each two groups, it was found that there were no statistically significant differences between the Negative control, CSF, LV, CSF-LV and STZ groups in the expression of any of the studied miRNAs.

However, there was a statistically significant elevation in the expression levels of all studied miRNAs in the Letrozole group compared with the Negative control, CSF, LV, CSF-LV and STZ groups. There was also a statistically significant elevation in the expression levels of all studied miRNAs in the STZ-L group compared with the Negative control, CSF, LV, CSF-LV and STZ groups. Finally no difference was found between Letrozole group and STZ-L group in the expression level of any of the studied miRNAs.

Correlation between memory status (in alternation percentage), p-Tau concentration and Let-7 miRNAs expression (in fold change) among different studied groups

Table 6 shows that there were statistically significant negative correlations between alternation percentage and the expression levels of Let-7a ($P=0.01$), Let-7b ($P=0.002$) & Let-7e ($P<0.001$) and p-Tau concentration ($P<0.001$). In addition, there were statistically significant positive correlation between p-Tau concentration and the expression levels of Let-7a ($P<0.001$), Let-7b ($P<0.001$) & Let-7e ($P<0.001$).

Discussion

AD causes aging-related cognitive impairment and dementia (1). Amnesic presentations as well as the presence of Aβ plaques and fibrillary tangles of Tau proteins are required for diagnosis [34, 35]. Studies have shown that the cerebral glucose mobilization disorders either precede or accompany the initial cognitive impairments in sporadic AD [36, 37].

Multiple studies provide evidence that Let-7 family of MicroRNA is neurotoxic and possibly involved in AD. These studies include models of $A\beta_{40}$-incduced neurotoxicity in cell lines [8], injection of Let-7b into the CSF of wild-type mice [6], quantification of Let-7 miRNAs in the CSF of AD patients [5], miRNA profiling of the brains of transgenic AD mice [38, 39], and studying the expression of miRNAs in the brains of a cholesterol-based rabbit model of AD [3].

Multiple drugs were found to increase the expression of Let-7 in various cell lines [9, 10], an example of which is Letrozole [11, 12], which is a potent aromatase inhibitor that can cross the blood brain barrier [13]. Controversial data exist regarding the effects of aromatase inhibitors (AIs), including Letrozole, on memory in rodents [14–17].

Prior to this current study, levels of Let-7 miRNAs were not investigated in an STZ-induced sporadic AD (sAD)-like model, neither was the possible effect of Let-7 modulation on the memory of the animals in such model. Results of the present work showed that STZ, Letrozole and STZ + Letrozole administration were all associated with memory impairments in rats, as evident by decreases in the alternation percentages of these groups compared to the control groups. The degree of memory impairment was more severe in the STZ + Letrozole rats, followed by the STZ group, then by the Letrozole group, which showed the least degree of memory impairment.

These results are partially in line with Nayebi et al. [14] whose results also showed a significant memory impairment in the STZ, Letrozole and STZ + Letrozole

Table 2 Let-7 miRNAs expression (in fold change) in the studied groups

Groups	Parameter		
	Let-7a fold change **Mean ± SD** **Median** **Range**	**Let-7b fold change** **Mean ± SD** **Median** **Range**	**Let-7e fold change** **Mean ± SD** **Median** **Range**
Negative control ($n=6$)	1.30 ± 1^{a} 0.87 0.46–2.59	1.55 ± 1.34^{a} 1.29 0.2–3.63	1.94 ± 1.74^{a} 1.75 0.9–4
CSF ($n=6$)	1.60 ± 1^{a} 1.57 0.67–2.57	1.8 ± 1.24^{a} 1.92 0.52–2.99	1.56 ± 0.74^{a} 1.89 0.62–2.36
LV ($n=6$)	1.35 ± 0.43^{a} 1.18 0.95–1.9	1.52 ± 0.32^{a} 1.53 1.16–1.88	1.70 ± 1.20^{a} 1.86 0.35–2.91
CSF-LV ($n=6$)	1.26 ± 2.45^{a} 0.21 0.17–6.25	0.84 ± 0.82^{a} 0.43 0.34–2.41	1.35 ± 2.35^{a} 0.41 0.11–6.11
STZ ($n=9$)	0.97 ± 0.84^{a} 0.64 0.06–2.45	1.89 ± 1.41^{a} 1.81 0.39–4.85	2.35 ± 1.60^{a} 2.91 0.47–5.54
Letrozole ($n=9$)	4.70 ± 2.07^{b} 5.18 0.8–7.85	6.47 ± 3.39^{b} 5.85 1.07–12.37	9.01 ± 5.12^{b} 7.36 2.89–17.27
STZ-L ($n=9$)	5.91 ± 3.94^{b} 4.8 3.1–14.25	5.93 ± 2.66^{b} 4.82 3.16–10.11	9.29 ± 6.28^{b} 7.73 3.2–19.97
KW	29.89	30.24	31.35
P	< 0.001**	< 0.001**	< 0.001**

KW: Kruskal–Wallis test, **Highly significant ($P<0.01$)

Groups with different letters have statistically significant ($P<0.05$) differences

Table 3 LSD for Let-7a fold change among different studied groups

	CSF ($n=6$)	**LV ($n=6$)**	**CSF-LV ($n=6$)**	**STZ ($n=9$)**	**Letrozole ($n=9$)**	**STZ-L ($n=9$)**
Negative control ($n=6$)	0.47 NS	0.63 NS	0.11 NS	0.35 NS	0.005**	0.001**
CSF ($n=6$)		0.99 NS	0.06 NS	0.09 NS	0.005**	0.001**
LV ($n=6$)			0.06 NS	0.41 NS	0.01*	0.001**
CSF-LV ($n=6$)				0.24 NS	0.02*	0.02*
STZ ($n=9$)					0.001**	< 0.001**
Letrozole ($n=9$)						0.86 NS

NS: Non significant ($P>0.05$), *Significant ($P<0.05$), **Highly significant ($P<0.01$)

Table 4 LSD for Let-7b fold change among different studied groups

	CSF ($n=6$)	**LV ($n=6$)**	**CSF-LV ($n=6$)**	**STZ ($n=9$)**	**Letrozole ($n=9$)**	**STZ-L ($n=9$)**
Negative control ($n=6$)	0.63 NS	0.63 NS	0.75 NS	0.56 NS	0.007**	0.003**
CSF ($n=6$)		0.99 NS	0.06 NS	0.48 NS	0.005**	0.001**
LV ($n=6$)			0.06 NS	0.56 NS	0.01*	0.001**
CSF-LV ($n=6$)				0.08 NS	0.002**	0.001**
STZ ($n=9$)					0.004**	0.002**
Letrozole ($n=9$)						0.63 NS

NS: Non significant ($P>0.05$), *Significant ($P<0.05$), **Highly significant ($P<0.01$)

Table 5 LSD for Let-7e fold change among different studied groups

	CSF ($n=6$)	LV ($n=6$)	CSF-LV ($n=6$)	STZ ($n=9$)	Letrozole ($n=9$)	STZ-L ($n=9$)
Negative control ($n=6$)	0.75 NS	0.99 NS	0.63 NS	0.56 NS	0.003**	0.01*
CSF ($n=6$)		0.75 NS	0.11 NS	0.29 NS	0.001**	0.001**
LV ($n=6$)			0.34 NS	0.18 NS	0.002**	0.001**
CSF-LV ($n=6$)				0.06 NS	0.003**	0.005**
STZ ($n=9$)					0.002**	0.001**
Letrozole ($n=9$)						0.72 NS

NS: Non significant ($P>0.05$), *Significant ($P<0.05$), **Highly significant ($P<0.01$)

Table 6 Correlations between alternation percentage, p-Tau concentration and Let-7 miRNAs expression

	Alternation percentage		p-Tau concentration	
	r	*P*	*r*	*P*
Let-7a fold change	−0.36	0.01*	0.57	< 0.001**
Let-7b fold change	−0.43	0.002**	0.62	< 0.001**
Let-7e fold change	−0.57	< 0.001**	0.64	< 0.001**
p-Tau concentration	−0.72	< 0.001**	–	–

r: Spearman correlation coefficient; *Significant ($P<0.05$); **Highly significant ($P<0.01$)

groups compared to the controls. However, while their results indicated that the Letrozole group had the least severe impairment among the three groups, there was no significant difference in the degree of memory impairment in the STZ group compared to the STZ + Letrozole group.

This discrepancy between our results and theirs may arise from: (1) Differences in the animal strains used, as the Wistar strain was used in their study, (2) The dose of Letrozole given and the administration route, as they used a dose of 6 mg/kg administrated intraperitoneally, or (3) The use of a different memory test; the passive avoidance test. Passive avoidance and T-maze tests were shown to give different results when testing the effects of other drugs on memory processes [40].

Regarding the level of p-Tau (Ser396), the results of our study showed a significant increase of p-Tau in the Letrozole, STZ and STZ + Letrozole groups compared to the control groups. While the ICV–STZ associated generation of hyperphosphorylated Tau and Tau pathology similar to that of AD was extensively documented in literature and previous studies [41–47], the possible effect of Letrozole on hyperphosphorylated Tau levels is underexplored; however, earlier studies showed that estrogen prevents hyper-phosphorylation of Tau protein in neuroblastoma cells as well as rodent cortical neuron cultures [48], an effect that might be reversed by an aromatase inhibitor, such as Letrozole, and that ovariectomy, which also reduces estrogen levels, increases age-related hippocampal Tau hyper-phosphorylation [49].

Hippocampal levels of p-Tau showed a significant negative correlation with the alternation percentages in the current study, which was postulated; as there is an evidence showing that both plasma and CSF levels of Tau correlate with the incidence of AD dementia in patients [50], and that CSF Tau levels have an inverse correlation with the short-term memory scores in patients with AD [51].

Regarding the expression levels of Let-7 a, b and e, miRNAs, we are first to report the expression patterns of these three miRNAs in an STZ-induced sAD-like rat model. Let-7a, b and e miRNAs were not differentially expressed in the STZ group compared to the controls.

Multiple studies have examined the expression levels of various Let-7 miRNAs in different models, samples, and time intervals, yielding different results. For example, studies examining Let-7 members in the CSF of AD patients—of different subtypes—have found Let-7a-3p, b, e, f and i to be upregulated [5, 52–54], while Let-7a was found to be downregulated in the CSF of patients with late onset AD [55]. Furthermore, studies examining Let-7 levels in the serum or plasma of AD and Mild cognitive impairment (MCI) patients have found Let-7f to be upregulated in AD [56], and Let-7b to be upregulated in MCI [57], while Let-7d, g and i were downregulated [58–60]. On the other hand, Animal studies on different transgenic AD mice and cholesterol-induced AD rabbits have found Let-7a, b, c, d, e and f to be upregulated in the brain at different time intervals [3, 38, 39]. Thus, it was not unexpected that the model chosen for the current study might show different results, since none of the previous studies, to date, have examined the levels of Let-7 in a similar STZ-induced AD rodent model. It may also be assumed that measuring expression levels of Let-7 in aged STZ-induced AD rats might yield different results than the ones obtained from this current study, as we only examined the hippocampal levels of selected Let-7 miRNAs after 3 weeks of STZ injection, i.e., during the acute impairment stage of this model.

Non-significant differences in the expression of Let-7 a, b and e in the STZ group do not necessarily mean that these miRNAs are not implicated in the disease process in this model. Let-7 activity was shown to be regulated by another miRNA, miR-107. miR-107 directly interacts with Let-7, suppresses its function and promotes its degradation [61]. miR-107 was found to be downregulated in AD in multiple studies [7, 62–64]. It was also found to be deregulated in STZ-induced diabetic animals [65]. Decreases in miR-107 levels or activity can lead to Let-7 stabilization and preservation of its action on the target genes [66]. However, further research is needed to elucidate the relation between Let-7 and miR-107 in AD.

On the other hand, Letrozole administration was associated with Let-7a, b and e upregulation in the Letrozole and the STZ + Letrozole groups, which showed significant differences in Let-7a, b and e expression levels compared to all other groups. No significant difference was found in the expression of Let-7a, b or e between the Letrozole group and the STZ + Letrozole group, further serving to possibly relate the observed upregulation to the action of Letrozole.

The observed effect of Letrozole on Let-7 miRNAs was not unexpected, as Letrozole was shown to be able to cross the blood brain barrier [13, 67], and it was previously shown to be associated with increased expression of various Let-7 miRNAs, including Let-7f, b and a, in various cancer models [11, 12, 68].

Analysis of the current data showed significant correlations between Let-7a, b and e levels and both the alternation percentage and p-Tau level in the hippocampus. There was a significant negative correlation between the expression level of each of the Let-7 miRNAs tested (Let-7a, b and e) and the alternation percentage, and a significant positive correlation between the expression level of each of the Let-7 miRNAs tested and the hippocampal p-Tau level.

Studies exploring possible correlations between Let-7 members and either or both cognitive impairment and p-Tau levels are scarce, and majorly differ from the current work in regards to the type of sample, and the type of cognitive test/exam which is determined by the species to be examined. For instance, Kenny et al. [57] found no significant correlation between Let-7b expression and the cognitive decline in MCI patients, and Derkow et al. [5] did not observe a significant correlation between amounts of $A\beta_{42}$ or total-Tau and Let-7b or Let-7e expression in the CSF of AD patients. Further research is needed to establish more evidence in regards to Let-7 correlation with memory status and p-Tau levels, preferably using larger samples and different AD models.

Based on the findings of this current study, Letrozole alone was associated with a significant degree of memory impairment, but, the more severe impairment in the STZ + Letrozole group could further support the idea of a synergistic effect exerted by Letrozole in presence of an additional pathological insult, a finding that is in agreement with a previous study that found evidence of synaptic pathology and changes in mitochondrial morphology in hippocampal cultures treated with both Letrozole and Aβ, suggesting that Letrozole potentiates the pathological effects of Aβ [69]. However, this current study cannot confirm whether this effect is due to the neurosteroidal deficit caused by Letrozole or its modulation of some neurotoxic miRNAs of the Let-7 family. Another limitation of this study lies in the fact that correlations alone, while they may indicate a relationship, cannot be used to suggest a causal relationship. Further experimental studies are needed to confirm the presence of a relationship, such as overexpression or knockout studies of Let-7 miRNAs.

Upregulated Let-7 could play a role in the pathogenesis of AD by affecting multiple pathways that relate to insulin signaling and neuroinflammation, and are known to be disturbed by STZ administration as well as being regulated by estrogen. STZ can accumulate via Glucose transporter 2 (GLUT2) uptake [70], and cause decreased mRNA expression and membrane protein levels of insulin receptors (IR), and insulin-like growth factor-1 receptors (IGF-1R) in hippocampal cells [71]. Insulin receptor substrates (IRSs) and protein kinase B (Akt) phosphorylation (i.e. activation) also decrease, resulting in disruption of the insulin signaling pathway, which was implicated in the process of learning and memory [72]. This impairment leads to activation of Glycogen synthase kinase 3 (GSK3), ultimately resulting in hyperphosphorylation of Tau protein [47] and promoting Aβ accumulation [73]. STZ also upregulates Beta-secretase 1, which is also known as beta-site amyloid precursor protein cleaving enzyme 1 (BACE1), which can result in increased cleavage of amyloid precursor protein [47]. Let-7 was also found to target the mRNAs of the insulin signaling pathway players, such as IR, IGF1R, IRS, Phosphatidylinositol-3-kinase (PI3K) and Akt [74, 75] and Let-7 transfection into a human cell line led to reductions in IR and IRS [75].

We suggest that it is possible the upregulation of Let-7 in the hippocampus could aggravate the toxic effects of STZ via further dysregulation and inhibition of the insulin signaling pathway. This suggestion can be supported by a recent study that found that LIN-28a, which is a powerful inhibitor of Let-7 biogenesis, prevented the toxic effects of STZ on pancreatic β-cells, and that LIN-28a downregulation of Let-7 was associated with increased phosphorylation of PI3K and Akt in a mouse insulinoma cell line [76].

Interestingly, estrogen binding to G-coupled estrogen receptor (GPER) could result in the activation of the PI3K/Akt pathway [77, 78], which could lead to memory enhancement [79]. In addition, the estrogen/ER–PI3K–Akt pathway has been shown to be a neuroprotective factor against toxic insults to neuronal cells [80]. It might be possible that the estrogen-reducing effect of Letrozole could have also added to the inhibition of the PI3K–Akt pathway already caused by the STZ toxicity and the upregulation of Let-7.

Another pathway by which upregulation of Let-7 could aggravate the effect of an STZ-induced lesion is through increasing neuroinflammation. STZ leads to increased expression of neuroinflammation markers, such as NF-κB (Nuclear factor kappa light chain enhancer of activated B cells), and Glial fibrillary acidic protein (GFAP) positive astrocytes in the hippocampus [47, 81]. Increased expression and activation of astrocytes for prolonged periods of time is evident in AD brain, in which severity of glial activation correlated with cognitive decline [82]. The activation of astrocytes is accompanied by increased production of potentially neurotoxic factors including inflammatory cytokines, nitric oxide and reactive oxygen species [83].

Microglia express Toll-like receptors (TLRs), which play an important role in the immune response as they recognize pathogen molecules. TLRs activation leads to subsequent activation of intracellular signaling cascades that ultimately cause the release of inflammatory molecules [6, 84]. Let-7 is a potent activator of TLR7 signaling in microglia and neurons, leading to induction of NF-κB in microglia, an inflammatory response and initiation of neurodegeneration [6], which can add to the already existing pro-inflammatory effects of STZ, aggravating its neurodegenerative actions.

Conclusions

This study shows changes in the expression of Let-7a, b and e miRNAs in association with Letrozole administration, and correlations between the expression of the studied Let-7 miRNAs and both the status of working memory and the hippocampal p-Tau levels, further adding to the evidence of their possible neurotoxic effects. The association between Letrozole administration and the increased expression of some Let-7 miRNAs warrants further investigation into the possible effects of this highly used drug and other brain-related miRNAs, especially those of pathological importance.

Further studies are needed to examine the levels of expression of Let-7a, b and e miRNAs in the later, chronic, progressive stages of the STZ-induced sAD model, and to test the effects of increased expression of Let-7a, b and e miRNAs on their insulin signaling pathway-related targets.

Abbreviations

aCSF: Artificial cerebrospinal fluid; AD: Alzheimer's disease; AIs: Aromatase inhibitors; Akt: Protein kinase B; ANOVA: Analysis of variance; AP: Anteroposterior; Aβ: Beta-amyloid; BACE1: Beta-site amyloid precursor protein cleaving enzyme 1; BBB: Blood brain barrier; CA2: Cornu Ammonis 2; $CaCl_2$: Calcium chloride; CMC: Barboxy methylcellulose; DV: Dorsoventral; ELISA: Enzyme-linked immunosorbent assay; GFAP: Glial fibrillary acidic protein; GLUT2: Glucose transporter 2; GPER: G-coupled estrogen receptor; GSK3: Glycogen synthase kinase 3; H&E: Hematoxylin and Eosin; IACUC: Institutional Animal Care and Use Committee; ICV: Intracerebroventricular; IGF-1R: Insulin-like growth factor-1 receptors; IR: Insulin receptors; IRSs: Insulin receptor substrates; KCl: Potassium chloride; LSD: Least significant difference; MCI: Mild cognitive impairment; MERC: Medical Experimental Research Center; MgCl2: Magnesium chloride; miRNAs: MicroRNAs; ML: Mediolateral; NaCl: Sodium chloride; NaH2PO4: Monosodium dihydrogen orthophosphate; NaHCO3: Sodium bicarbonate; NF-κB: Nuclear factor kappa light chain enhancer of activated B cells; PBS: Phosphate-buffered saline; PI3K: Phosphatidyl-inositol-3-kinase; p-Tau: Hyperphosphorylated Tau; qRT–PCR: Quantitative real-time reverse transcription–polymerase chain reaction; RT: Reverse transcription; sAD: Sporadic Alzheimer's disease; SD: Standard deviation; STZ: Streptozotocin; TLRs: Toll-like receptors.

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Author contributions

NM, ME, SA and DA conceived and designed the study. NM and NH ran the experiments and provided reagents. NM, with the help of the acknowledged DE, analyzed data. NM wrote the manuscript then it was revised and edited by NH and DA. The authors declare that all data were generated in-house and that no paper mill was used. All authors read and approved the final manuscript.

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Availability of data and materials

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

An ethical approval for this study was obtained from the Institutional Animal Care and Use Committee (IACUC), Zagazig University (ZU) (Approval number: ZU-IACUC/3/F/83/2018) and animal experiments followed the ARRIVE guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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