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Comparative study of rivastigmine and galantamine on the transgenic *Drosophila* model of Alzheimer's disease



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ABSTRACT

Alzheimer's Disease (AD) is characterized as a progressive neurodegenerative disease most commonly associated with memory deficits and cognitive decline. The formation of amyloid plaques and neurofibrillary tangles are important pathological markers of AD. The accumulation of amyloid plaques and neurofibrillary tangles leads to the loss of neurons including the cholinergic neurons thus decreasing the levels of acetylcholine (a neurotransmitter). To reduce the AD symptoms cholinesterase inhibitors are widely used to decrease the hydrolysis of acetylcholine released from presynaptic neurons. In the present study we have studied the effect of rivastigmine and galantamine (commonly used cholinesterase inhibitors) on the transgenic *Drosophila* model of AD expressing human A β -42 in the neurons. The effect of similar doses of rivastigmine and galantamine (i.e. 0.1, 1 and 10 mM) was studied on the climbing ability, lifespan, oxidative stress markers, caspase 9 and 3, acetylcholinesterase activity and on the formation of A β -42 aggregates. The results suggest that the rivastigmine is more potent in reducing the oxidative stress and improving climbing ability of AD flies. Both the drugs were found to be effective in increasing the lifespan of AD flies. Galantamine was found to be a more potent inhibitor of acetylcholinesterase compared to rivastigmine. Galantamine prevents the formation of A β -42 aggregates more effectively compared to rivastigmine.

1. Introduction

Alzheimer's Disease (AD) is a progressive, degenerative brain disorder which results in cognitive as well as behavior decline and ultimately leads to death (Lockhart et al., 2009). During the progression of AD there is a profound loss of neurons including the cholinergic neurons leading to decline in the levels of acetylcholine (García-Ayllón et al., 2011). Acetylcholinesterase breaks down acetylcholine into acetic acid and choline. In this context the inhibitors of acetylcholinesterase are effective in maintaining the proper levels of acetylcholine and thus reducing the symptoms of AD (Marucci et al., 2021). For the treatment of mild to moderate AD symptoms, cholinesterase inhibitors such as donepezil, rivastigmine and galantamine are used and for the treatment of moderate to severe AD symptoms N-methyl-D-aspartate antagonist memantine is prescribed (Bond et al., 2012). The efficacy of these drugs has been studied in a number of clinical studies (Li et al., 2019; Onor et al., 2007) and in various experimental models (Van Dam et al., 2005; Bezerra da Silva et al., 2016; Mohamed et al., 2015). Kröger et al. (2015) has reported that the use of rivastigmine and galantamine is associated with an

increased risk of cardiac events. The systematic review by Clegg et al. (2002) suggest that the donepezil, rivastigmine and galantamine were beneficial in treating various stages of AD, but the implications of the use of donepezil, rivastigmine and galantamine were unclear. No clear evidence exists to date which one of these drugs is more efficacious (Hansen et al., 2008). The comparative study by Aguglia et al. (2004) and Spencer and Noble (1998) showed that there was no statistical difference between the three drugs at three months of treatment. Galantamine hydrobromide, is a tertiary alkaloid. It is not only a reversible competitive inhibitor of acetylcholinesterase, but also act as an allosteric modulator of nicotinic acetylcholine receptors (Zarotsky et al., 2003). Rivastigmine is a carbamylating, long-acting reversible and non-competitive carbamate acetylcholinesterase inhibitor (Spencer and Noble, 1998). Oxidative stress has been proposed as one of the factors that plays a vital role in the pathogenesis various neurodegenerative disorders such as Amyotrophic Lateral Sclerosis (ALS), Parkinson's disease (PD) and AD (Niedzielska et al., 2016). It has been reported to cause the death of neuronal cells not only in transgenic mice model for AD but also in AD patients (Butterfield Allan, 2002; Resende et al., 2008).

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Drosophila melanogaster due to the fact that 70% of disease-associated human genes have a fly homolog is the best suited invertebrate model to study the pathogenesis of neurodegenerative disorders (Bier, 2005; Lenz et al., 2013). The genome of *Drosophila* is well defined and its culture also offer low cost maintenance due to which this model provides good platform to study the pathology of several diseases. The brain of *Drosophila* is easily accessible which allows easy image capturing and quantification of amyloid plaque deposition in transgenic models along with the study of various cognitive parameters (Uras et al., 2021). The newly synthesized acetylcholinesterase inhibitor XJP-1, resulted in a significant improvement of AD symptoms and reduction of amyloid plaques by reducing the amyloid aggregation in transgenic *Drosophila* model of AD (Uras et al., 2021). XJP-1, also improves the climbing ability and life span of the transgenic flies expressing A β -42 (Uras et al., 2021).

There is also a reasonable similarity between the central nervous systems of flies and humans with both consisting of neurons, glia and utilizes the same neurotransmitters (Lenz et al., 2013). At present number of transgenic *Drosophila* models are available to study the various aspects of neurodegenerative diseases such as PD, AD and Huntington's Disease (HD) (Marsh and Thompson, 2006; Bilen and Bonini, 2005). Among these transgenic models, one model express human A β -42 under GAL4-UAS system in the brain of flies. Such flies exhibit diffuse amyloid deposits, age dependent loss in climbing ability, memory, olfaction and neurodegeneration (Lu and Vogel, 2009; Beg et al., 2018; Ali et al., 2019). Since AD is associated with the decline motor activity, memory loss, deposition of A β -42 and increase in oxidative stress hence, we decided to study the effect of rivastigmine and galantamine on lifespan, climbing ability and oxidative effect stress markers. Immunohistochemistry was also performed on the brain sections to study the effect of rivastigmine and galantamine on the expression of A β -42 aggregates.

2. Materials and methods

***Drosophila* stocks.** Transgenic fly lines expressing wild-type human *Abeta42* "*w*[1118];*P*{*w*[+*mC*] = *UASAPP.Abeta42.B*}*m26a* under UAS control and *GAL4*"*w*[*];*P*{*w*[+*mC*] = *GAL4-elavL*}*3* were obtained from Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN). When the males of UAS (Upstream Activation Sequence)-APP.Abeta42.B strains were crossed with the females of GAL4-elav. L (*vice-versa*) the progeny express human A β -42 in the neurons and the flies are referred as AD flies (Prubing et al., 2013).

***Drosophila* culture and crosses.** The flies were cultured on standard *Drosophila* food containing agar, corn meal, sugar and yeast at 25 °C (24 \pm 1) (Siddique et al., 2014). Crosses were set up as described in our earlier published work (Ali et al., 2011). The AD flies were allowed to feed separately on different doses of rivastigmine and galantamine mixed in the diet for 30 days. The doses were selected according to the method described by Fatima et al. (2017) (Fatima et al., 2017) The LD 50 for both Rivastigmine and galantamine was calculated. For rivastigmine and galantamine the LD 50 was 50 mM and 60 mM, respectively. The highest tested dose was kept less than 1/4th of the LD 50. In order to get a comparative account of the drugs the doses were kept similar for both the drugs i.e., 0.1, 1 and 10 mM.

***Drosophila* life span determination.** Newly eclosed flies from each treated as well as control groups were placed in the culture tubes (10 flies per tube; 3 replicates/treatment) containing desired concentration of the drugs. The flies were transferred to a new diet at every 3rd day containing desired concentration of the drugs till the last one died (Long et al., 2019).

***Drosophila* climbing assay.** The climbing assay was performed as described by Pendleton et al. (2002). Ten flies were placed in an empty glass vial (10.5 cm \times 2.5 cm). A horizontal line was drawn 8 cm above the bottom of the vial. After the flies had acclimated for 10 min at room temperature, both controls and treated groups were assayed at random to a total of 10 trials for each. The mean values were calculated and then averaged and a group mean and standard error were obtained.

Preparation of homogenate for biochemical assays. Fly heads from each group were isolated (100 heads/group; five replicates/group) and the homogenate was prepared in 0.1 M phosphate buffer for the biochemical assays. Before performing the biochemical assays, the protein content in the homogenate was estimated by the method of Lowry (Waterborg and Matthews, 1994).

Estimation of glutathione (GSH) content. GSH content was estimated colorimetrically using Ellman's reagent (DTNB) according to the procedure described by Jollow et al. (1974). The assay mixture consisted of 550 μ l of 0.1 M phosphate buffer, 100 μ l of supernatant and 100 μ l of DTNB. The OD was read at 412 nm and the results were expressed as μ mol of GSH/gram tissue.

Estimation of glutathione-S-transferase (GST) activity. The GST activity was determined by the method of Habig et al. (1974). The reaction mixture consisted of 500 μ l of 0.1M phosphate buffer, 150 μ l of 10 mM CDNB, 200 μ l of 10 mM reduced glutathione, and 50 μ l of supernatant. The OD was taken at 340 nm and the enzyme activity was expressed as μ mol of CDNB conjugates formed/min/mg protein.

Lipid peroxidation assay. Lipid peroxidation was measured according to the method described by Ohkawa et al. (1979). 5 μ l of 10 μ M butylhydroxy toluene (BHT), 200 μ l of 0.67% thiobarbituric acid (TBA), 600 μ l of 1% orthophosphoric acid (OPA), 105 μ l of distilled water and 90 μ l of the sample were taken in an eppendorf, vortexed and kept in the water bath at 90 °C for 45 min. OD was read at 535 nm and the results were expressed in μ mol of TBARS formed/60 min/g tissue.

Estimation of protein carbonyl content (PCC). The PCC was estimated according to the protocol described by Hawkins et al. (2009). The homogenate was diluted to a protein concentration of approx 1 mg/ml. About, 250 μ l of diluted homogenate was taken in eppendorf. To it, 250 μ l of 10 mM 2,4-dinitrophenyl hydrazine (dissolved in 2.5 M HCl) was added, vortexed and kept in dark for 20 min. About 125 μ l of 50% (w/v) trichloroacetic acid (TCA) was added, mixed thoroughly and incubated at -20 °C for 15 min. The tubes were then centrifuged at 4 °C for 10 min at 8200g. The supernatant was discarded and the pellet obtained was washed twice by ice cold ethanol:ethyl acetate (1:1). Finally, the pellets were re-dissolved in 1 ml of 6 M guanidine hydrochloride and the absorbance was read at 370 nm.

Determination of catalase (CAT) activity. The catalase activity was estimated according to the method of Beers and Sizer (1952) by kinetic method where rate of dismutation of H₂O₂ to water and molecular oxygen is proportional to the concentration of catalase in the sample. The reaction mixture consisted of 650 μ l of 0.1 M phosphate buffer, 333 μ l of H₂O₂ (0.05M) and 17 μ l of sample. A decrease in OD was measured for 2 min, at 30 s intervals at 240 nm. The activity of catalase was calculated and expressed as μ mol of H₂O₂ consumed/min/mg protein.

Determination of superoxide dismutase activity (SOD) activity. The SOD activity was estimated according to the method of Marklund and Marklund (1974). The reaction mixture consisted of 17 μ l of sample and 950 μ l of 0.1M phosphate buffer. The reaction was initiated by adding pyrogallol. An increase in OD was noted at 420 nm for 3 min at 30 s interval and the results were expressed as units/mg protein.

Caspase-3 (Drice) and Caspase-9 (Dronc) activities. The assay was performed according to the manufacturer protocol (Bio-Vision, CA, USA). The assay was based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) obtained after specific action of caspase-3 and caspase-9 on tetrapeptide substrates, DEVD-pNA and LEHD-pNA, respectively. The assay mixture (50 μ l of homogenate and 50 μ l of chilled cell lysis buffer) was incubated on ice for 10 min. After incubation, 50 μ l of 2X reaction buffer (containing 10 mM DTT) with 200 μ M substrate (DEVD-pNA for Drice, and IETD-pNA for Dronc) was added and incubated at 37 °C for 1.5 h. The reaction was quantified at 405 nm.

Acetylcholinesterase (AChE) activity. Acetylcholinesterase activity was determined by the method described by Ellman et al. (1961). It is based on the principle that AChE hydrolyses the acetylthiocholine to produce thiocholine and acetate. The thiocholine in turn reduces the Dithiobis-nitrobenzoate liberating nitrobenzoate, which absorbs light at

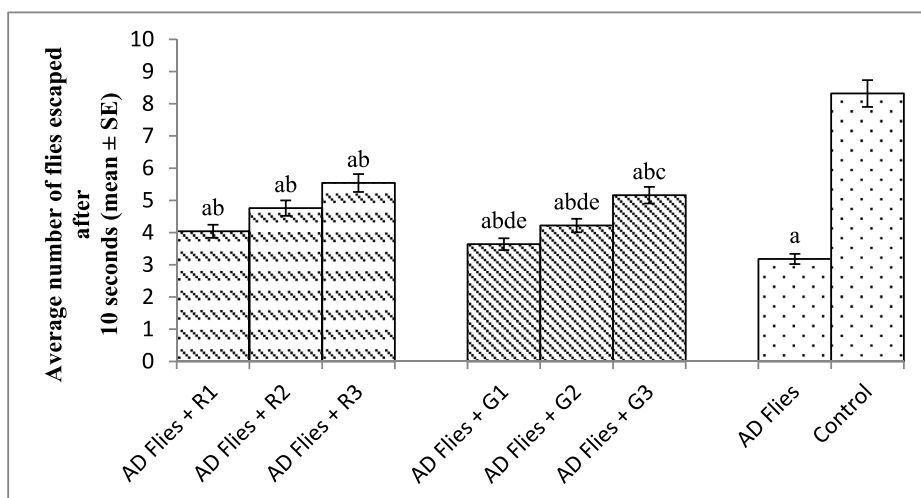


Fig. 1. Effect of Rivastigmine (R) and Galantamine (G) on the climbing ability ($df = 39$; $F = 154.1$) on AD Flies. The flies were allowed to feed on the diet supplemented with various doses of R and G and then assayed. [a-significant with respect to control, $p < 0.05$; b-significant with respect to AD flies, $p < 0.05$; c-significant between R1 and G1,G2,G3 at $p < 0.05$; d-significant between R2 and G1,G2,G3 at $p < 0.05$; e-significant between R3 and G1,G2,G3 at $p < 0.05$] [AD: Alzheimer's Disease; R1, G1 = 0.1, R2, G2 = 1 R3, G3 = 10 mM].

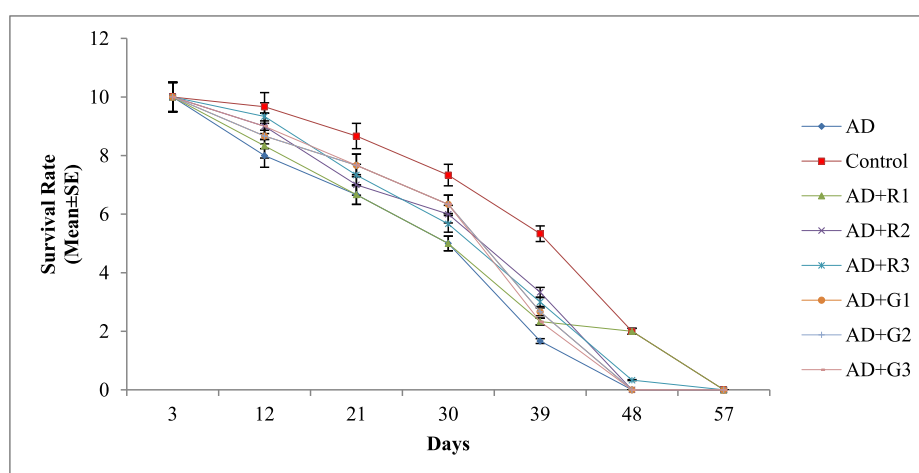


Fig. 2. Effect of Rivastigmine (R) and Galantamine (G) on the life span of AD flies. The flies were allowed to feed on the diet supplemented with various doses of R and G till the last fly died. [AD: Alzheimer's Disease R1, G1 = 0.1, R2, G2 = 1 R3, G3 = 10 mM; {3rd Day ($df = 23$; $F = 0$), 12th Day ($df = 23$; $F = 0$), 21st Day ($df = 23$; $F = 2.262$), 30th Day ($df = 23$; $F = 1.929$), 39th Day ($df = 23$; $F = 15.937$), 48th Day ($df = 39$; $F = 27.974$) and 57th Day ($df = 23$; $F = 0$)}.]

412 nm. The assay is based on measurement of the change in absorbance at 412 nm. The experiment was initiated with the reaction mixture consisting of 100 μ l of the sample, 650 μ l of 0.1 M phosphate buffer and 100 μ l of 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB). To the mixture 10 μ l of acetylthiocholine was added and the change in OD was recorded at every 1 min interval for 3 min.

Immunohistochemistry. The fly heads were isolated and the paraffin sections were prepared according to the procedure described by Palladino et al. (2002). The sections were deparaffinized and rehydrated. The slides were blocked in 8% Bovine Serum Albumin (BSA) for 2.5 h. Then the slides were washed with phosphate buffer saline (pH 7.2) containing 2% BSA for 5 min. For immunohistochemistry, after washing the slides were incubated with primary antibody (Rabbit monoclonal A β 42 antibody, Merck; 1:200 dilutions) in a humidified chamber for 12 h at 4 °C. The slides were then washed with PBS containing 2% BSA for 5 min and incubated with secondary antibody (Goat anti-Rabbit IgG, alkaline phosphatase conjugate, Merck, USA) at room temperature for 2 h. The final wash was given by PBS containing 2% BSA for 5 min. 5-Bromo-4-chloro-3-indolyl phosphate-Nitro blue tetrazolium chloride (BCIP-NBT) was used as a chromogenic substrate which interacts with secondary antibody to produce blue coloured product. The slides were then mounted in DPX and observed under the microscope. The A β 42 aggregates were quantified in terms of A β -positive cells using Image-J software.

Statistical analysis. The data was analyzed by one-way analysis of variance (ANOVA) followed by post hoc Tukey test using GraphPad Prism software [version 5.0]. The level of significance was kept at $p < 0.05$. The results were expressed as mean \pm SEM.

3. Results

3.1. Climbing assay

The AD flies showed a significant decrease of 2.61-fold in the climbing ability compared to control flies (Fig. 1; $p < 0.05$). The AD flies exposed to 0.1, 1 and 10 mM of rivastigmine showed a significant increase of 1.27, 1.49 and 1.74 folds, respectively, in the climbing ability compared to AD flies. The AD flies exposed to 0.1, 1 and 10 mM of galantamine showed a dose dependent significant increase of 1.14, 1.32 and 1.62 folds, respectively, in the climbing ability compared to AD flies (Fig. 1; $p < 0.05$).

3.2. Life span

The results obtained for the life span is shown in Fig. 2. The analysis on the 3rd day reveal no significant difference between AD flies, AD flies exposed to rivastigmine as well as galantamine and control flies ($F = 0$; $df = 23$; $p < 0.05$). The analysis on the 12th day also reveal the similar

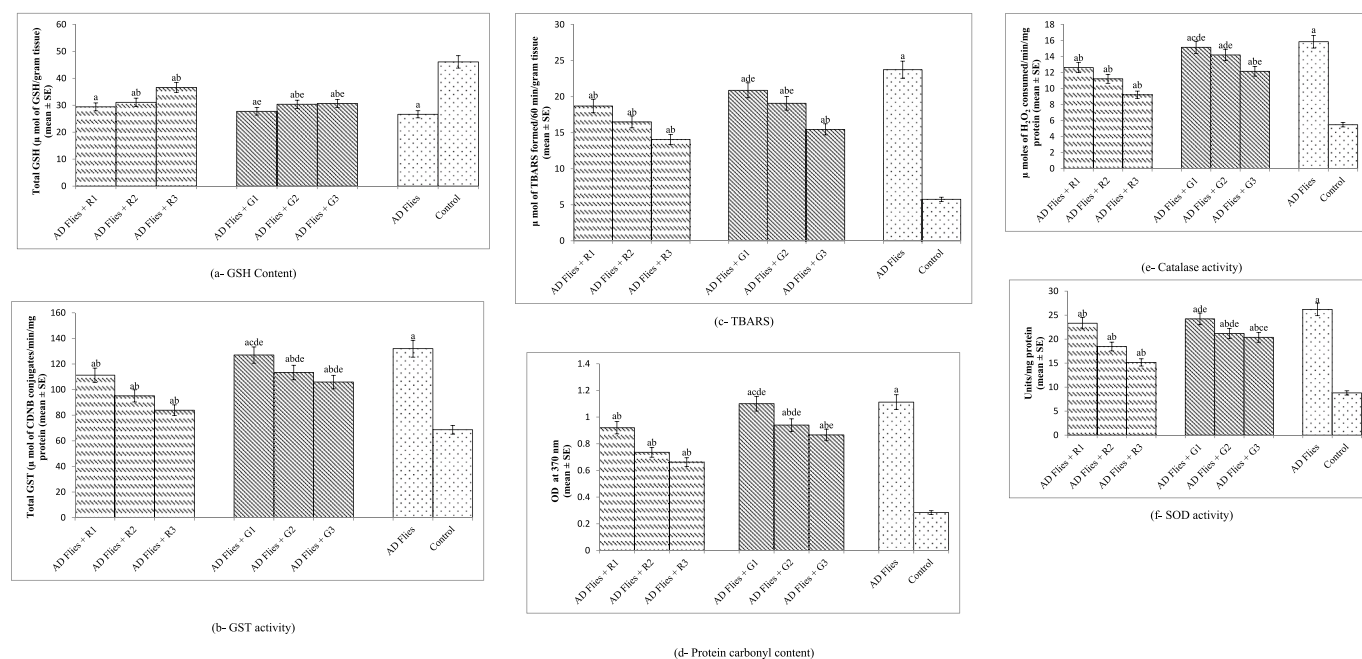


Fig. 3. Effect of Rivastigmine (R) and Galantamine (G) on the Glutathione (GSH) ($df = 39$; $F = 62.45$) (a), Glutathione-S-Transferase activity (GST) ($df = 39$; $F = 154.1$) (b), TBARS ($df = 39$; $F = 41.83$) (c), Protein Carbonyl content (PC) ($df = 39$; $F = 88.06$) (d), Catalase activity ($df = 39$; $F = 81.44$) (e) and Superoxide dismutase activity (SOD) ($df = 39$; $F = 100.0$) (f), in the brains of flies. The flies were allowed to feed on the diet supplemented with various doses of R and G and then assayed. [a-significant with respect to control, $p < 0.05$; b-significant with respect to AD flies, $p < 0.05$; c-significant between R1 and G1,G2, G3 at $p < 0.05$; d-significant between R2 and G1,G2, G3 at $p < 0.05$; e-significant between R3 and G1,G2,G3 at $p < 0.05$] [AD: Alzheimer's Disease; R1, G1 = 0.1, R2, G2 = 1 R3, G3 = 10 mM].

results ($F = 1$; $df = 23$; $p < 0.05$). The analysis on the 21st day also reveal no significant difference between AD flies, AD flies exposed to rivastigmine as well as galantamine and control flies ($F = 2.262$; $df = 23$; $p < 0.05$). The analysis on the 30th day reveal a significant difference in the survival rate of AD flies and control flies but no significant difference was observed between the AD flies exposed to rivastigmine as well as galantamine ($F = 1.929$; $df = 23$; $p < 0.05$). The AD flies showed a significant decrease of 1.47-fold in the survival rate compared to control flies (Fig. 2; $p < 0.05$). The analysis on the 39th day reveals a significant difference between AD flies, AD flies exposed to rivastigmine and control flies ($F = 15.937$; $df = 23$; $p < 0.05$). The AD flies showed a significant decrease of 3.59 folds in the survival rate compared to control flies ($p < 0.05$). The AD flies exposed to 1 and 10 mM of rivastigmine showed a significant increase of 2.79 and 2.39 folds in the survival rate compared to unexposed AD flies (Fig. 2; $p < 0.05$). No significant difference was observed among the flies exposed to 0.1 mM of rivastigmine and 0.1 and 10 mM of galantamine compared to unexposed AD flies ($p < 0.05$). On the 48th day all the unexposed AD flies died compared to control flies and the AD flies exposed 10 mM of rivastigmine ($F = 27.974$; $df = 23$; $p < 0.05$). In all remaining groups the flies were found to be dead on the 57th day (Fig. 2).

3.3. GSH content

The AD flies showed a significant decrease of 1.72-fold in GSH content compared to control flies (Fig. 3a; $p < 0.05$). The AD flies exposed to 1 and 10 mM of rivastigmine showed a dose dependent significant increase of 1.16 and 1.37 folds, respectively, in GSH content compared to unexposed AD flies ($p < 0.05$). The AD flies exposed to 1 and 10 mM of galantamine showed a dose dependent significant increase of 1.13 and 1.15 folds, respectively, in GSH content compared to unexposed AD flies ($p < 0.05$). No significant increase in GSH content was observed in AD flies exposed to 0.1 mM of rivastigmine and galantamine compared to unexposed AD flies (Fig. 3a).

3.4. GST activity

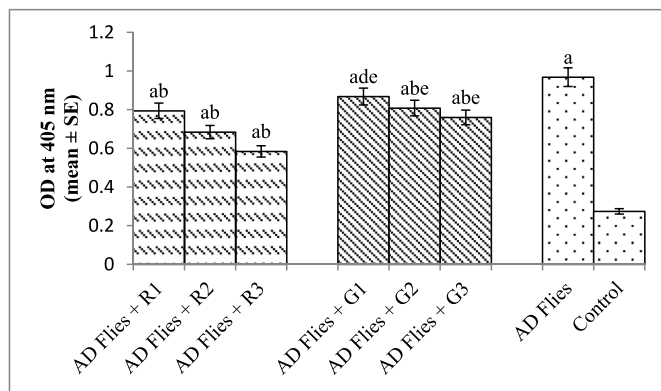
The AD flies showed a significant increase of 1.92-fold in the activity of GST compared to control flies (Fig. 3b; $p < 0.05$). The AD flies exposed to 0.1, 1 and 10 mM of rivastigmine showed a dose dependent decrease of 1.18, 1.38 and 1.57 folds, respectively, in the activity of GST compared to unexposed AD flies (Fig. 3b; $p < 0.05$). The AD flies exposed to 1 and 10 mM of galantamine showed a dose dependent decrease of 1.16 and 1.25 folds, respectively, in the activity of GST compared to unexposed AD flies ($p < 0.05$). The AD flies exposed to 0.1 mM of galantamine did not showed significant decrease in the activity of GST compared to unexposed AD flies (Fig. 3b).

3.5. TBARS

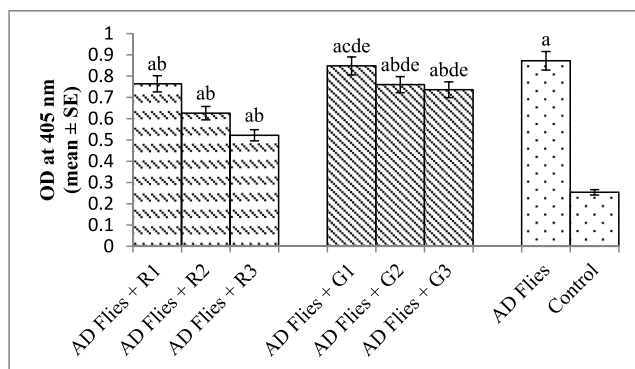
The AD flies showed a significant increase of 4.12-fold in TBARS compared to control flies (Fig. 3c; $p < 0.05$). A significant dose dependent decrease of 1.26, 1.44 and 1.69 folds in TBARS was observed in AD flies exposed to 0.1, 1 and 10 mM of rivastigmine, respectively, compared to unexposed AD flies ($p < 0.05$). The AD flies exposed to 1 and 10 mM of galantamine showed a significant decrease of 1.24 and 1.53 folds, respectively, in TBARS compared to unexposed AD flies ($p < 0.05$). The AD flies exposed to 0.1 mM of galantamine did not showed significant decrease in TBARS compared to unexposed AD flies (Fig. 3c; $p < 0.05$).

3.6. Protein carbonyl content

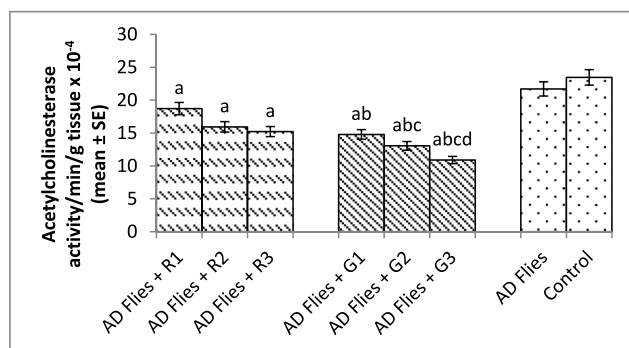
The AD flies showed a significant increase of 3.91-fold in the PC content compared to control flies (Fig. 3d; $p < 0.05$). The AD flies exposed to 0.1, 1 and 10 mM of rivastigmine showed a dose dependent significant decrease of 1.20, 1.51 and 1.68 folds, respectively, in the PC content compared to unexposed AD flies ($p < 0.05$). The AD flies exposed to 1 and 10 mM of galantamine showed a dose dependent significant decrease of 1.18 and 1.28 folds, respectively, in the PC content compared



(a)



(b)



(c)

Fig. 4. Effect of Rivastigmine (R) and Galantamine (G) on the Caspase-9 (df = 39; F = 42.83) (a), Caspase-3 (df = 39; F = 163.9) (b), Acetylcholinesterase (df = 39; F = 73.73) (c), in the brains of flies. The flies were allowed to feed on the diet supplemented with various doses of R and G and then assayed. [a-significant with respect to AD flies, $p < 0.05$; b-significant between R1 and G1,G2,G3 at $p < 0.05$; c-significant between R2 and G1,G2,G3 at $p < 0.05$; d-significant between R3 and G1,G2,G3 at $p < 0.05$] [AD: Alzheimer's Disease; R1, G1 = 0.1, R2, G2 = 1 R3, G3 = 10 mM].

to unexposed AD flies ($p < 0.05$). The AD flies exposed to 0.1 mM of galantamine did not showed significant decrease in the PC content compared to unexposed AD flies (Fig. 3d; $p < 0.05$).

3.7. Catalase activity

The AD flies showed a significant increase of 2.89-fold in the activity of catalase compared to control flies (Fig. 3e; $p < 0.05$). The AD flies exposed to 0.1, 1 and 10 mM of rivastigmine showed a significant decrease of 1.25, 1.41 and 1.72 folds, respectively, in the activity of catalase compared to unexposed AD flies ($p < 0.05$). The AD flies exposed to 10 mM of galantamine showed a significant decrease of 1.30-fold, in the activity of catalase compared to unexposed AD flies ($p < 0.05$). The AD flies exposed to 0.1 and 1 mM of galantamine did not showed significant decrease in the activity of catalase compared to unexposed AD flies (Fig. 3e; $p < 0.05$).

3.8. SOD activity

The AD flies showed a significant increase of 2.98-fold in the activity of SOD compared to control flies (Fig. 3f; $p < 0.05$). The AD flies exposed to 0.1, 1 and 10 mM of rivastigmine showed a significant decrease of 1.12, 1.41 and 1.72 folds, respectively, in the activity of SOD compared to unexposed AD flies ($p < 0.05$). The AD flies exposed to 1 and 10 mM of galantamine showed significant decrease of 1.23 and 1.28 folds, respectively, in the SOD activity compared to unexposed AD flies ($p < 0.05$). The AD flies exposed to 0.1 mM of galantamine did not showed significant decrease in the SOD activity compared to unexposed AD flies (Fig. 3f; $p < 0.05$).

3.9. Caspase-9 activity

The AD flies showed a significant increase of 3.53-fold in Caspase-9 activity compared to control flies (Fig. 4a; $p < 0.05$). The AD flies exposed to 0.1, 1 and 10 mM of rivastigmine showed a significant decrease of 1.21, 1.41 and 1.65 folds, respectively, in Caspase-9 activity compared to unexposed AD flies ($p < 0.05$). The AD flies exposed to 1 and 10 mM of galantamine showed a significant decrease of 1.19 and 1.27 folds, respectively in Caspase-9 activity compared to unexposed AD flies ($p < 0.05$). The AD flies exposed to 0.1 mM of galantamine did not showed significant decrease in caspase-9 activity compared to unexposed AD flies (Fig. 4a; $p < 0.05$).

3.10. Caspase-3 activity

The AD flies showed a significant increase of 3.43-fold in the Caspase-3 activity compared to control flies (Fig. 4b; $p < 0.05$). The AD flies exposed to 0.1, 1 and 10 mM of rivastigmine showed a significant decrease of 1.14, 1.39 and 1.67 folds, respectively, in Caspase-3 activity compared to unexposed AD flies ($p < 0.05$). The AD flies exposed to 1 and 10 mM of galantamine showed a significant decrease of 1.14 and 1.18 folds, respectively, in Caspase-3 activity compared to unexposed AD flies ($p < 0.05$). The AD flies exposed to 0.1 mM of galantamine did not showed significant decrease in caspase-3 activity compared to unexposed AD flies (Fig. 4b; $p < 0.05$).

3.11. Acetylcholinesterase activity

The AD flies showed no significant decrease in acetylcholinesterase activity compared to control flies (Fig. 4c; $p < 0.05$). The AD flies exposed to 0.1, 1 and 10 mM of rivastigmine showed a significant dose dependent decrease of 1.15, 1.36 and 1.42 folds, respectively, in acetylcholinesterase activity compared to unexposed AD flies ($p < 0.05$). The AD flies

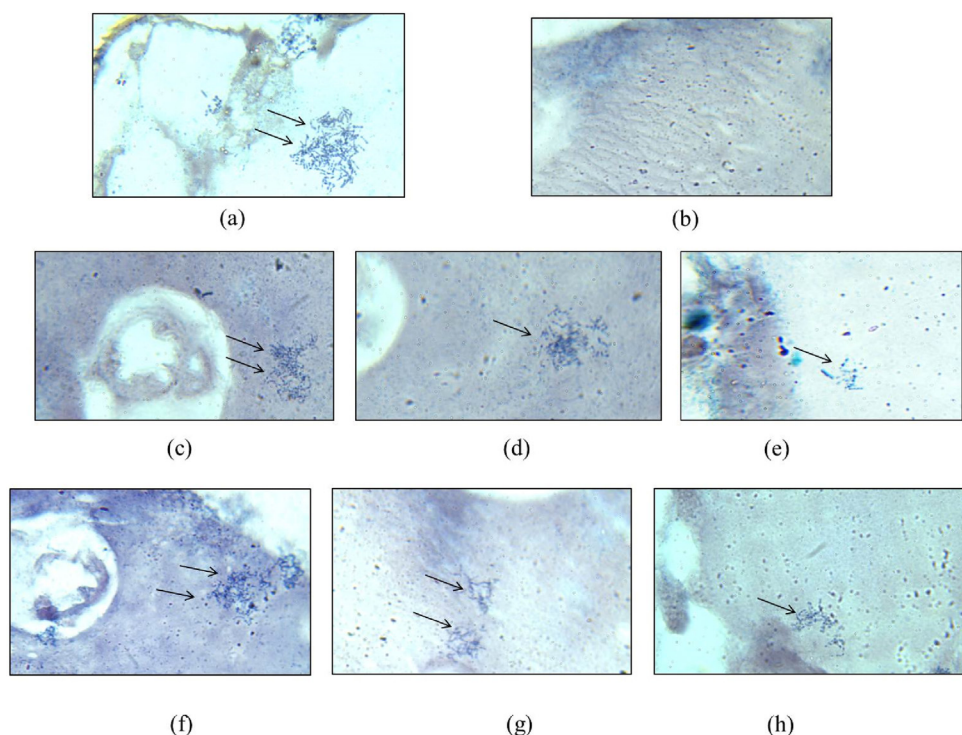
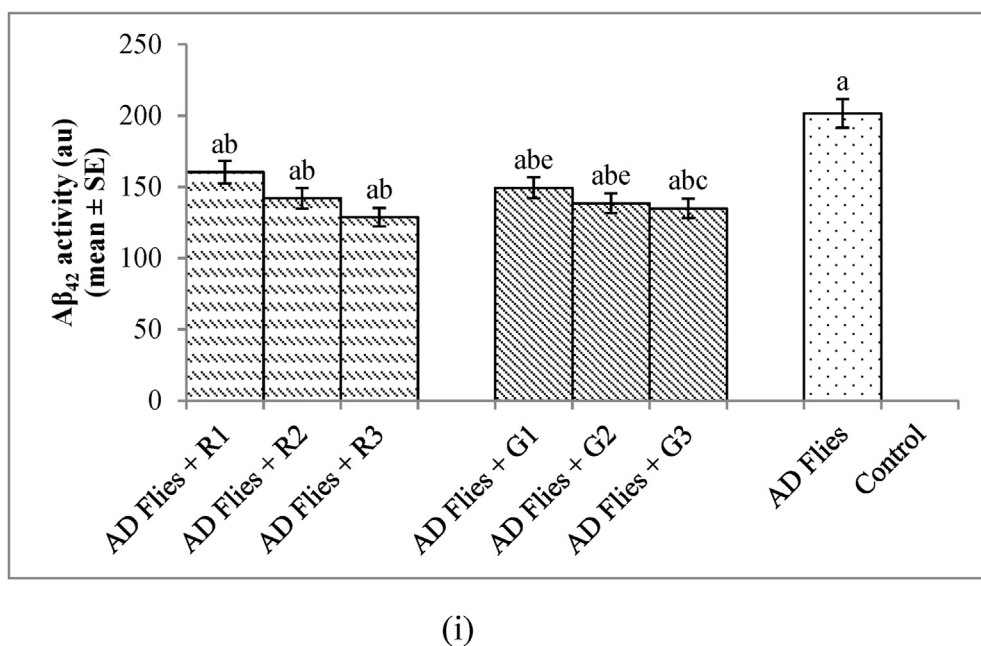


Fig. 5. A β -42 immunostaining (a–h) performed on the brain section of flies (N = 3) after 30 days of the exposure; a-AD fly, b-control, c-AD + R1, d- AD + R2, e- AD + R3, f-AD + G1, g- AD + G2, h- AD + G3 (100 X). i-Quantification of A β -42 aggregates from the total area of the brain using Image-J software (df = 25; F = 650.9). [a-significant with respect to control, p < 0.05; b-significant with respect to AD flies, p < 0.05; c-significant between R1 and G1,G2,G3 at p < 0.05; d-significant between R2 and G1,G2,G3 at p < 0.05; e-significant between R3 and G1,G2,G3 at p < 0.05] [AD: Alzheimer's Disease; R1, G1 = 0.1, R2, G2 = 1 R3, G3 = 10 mM].



exposed to 0.1, 1 and 10 mM of galantamine showed a significant dose dependent decrease of 1.46, 1.66 and 1.99 folds, respectively, in acetylcholinesterase activity compared to unexposed AD flies (Fig. 4c; p < 0.05).

3.12. Immunohistochemistry

The results obtained for immunohistochemistry are shown in Fig. 5(a–i). The AD flies showed a marked age dependent increase in the A β -42 aggregates compared to control flies (Fig. 5 a & b). The AD flies exposed to 0.1, 1 and 10 mM of rivastigmine showed a dose dependent significant decrease of 1.26, 1.42 and 1.56 folds, respectively, in the A β -

42 aggregates compared to the unexposed AD flies (Fig. 5i). The AD flies exposed to 0.1, 1 and 10 mM of galantamine also showed a significant dose dependent decrease of 1.34, 1.45 and 1.49 folds, respectively, in the A β -42 aggregates compared to the unexposed AD flies (Fig. 5i). The aggregates were quantified by using Image-J software.

4. Discussion

The results of the present study reveal that both the drugs i.e. rivastigmine and galantamine are effective in reducing the AD symptoms being mimicked in the transgenic *Drosophila*. Rivastigmine was found to be more effective in reducing the oxidative stress and improving the

climbing ability of AD flies compared to galantamine. Both the drugs were found to be almost equally effective in improving the life span of AD flies. Rivastigmine was found to be more effective in reducing the activity of Caspase-3 and 9, compared to galantamine, implying that rivastigmine is a more potent anti-apoptotic agent compared to galantamine. Galantamine inhibited the activity of acetylcholinesterase more effectively compared to rivastigmine thus exhibiting a more potent cholinesterase inhibitor compared to rivastigmine. The results obtained for immunohistochemistry reveals that both the drugs also prevent the formation of A β -42 aggregates in a dose dependent manner. Galantamine was found to be more effective in preventing the formation of A β -42 at lower doses.

The use of *Drosophila* and the negative geotaxis assay provides an inexpensive and reliable method to screen candidate drugs for phenotype rescue (Madabattula et al., 2015). In our present study both drugs significantly delayed the loss of climbing ability of AD flies but the effect was more significant in AD flies exposed to rivastigmine. Concerning their effect on the life span of AD flies both the drugs were effective in increasing the life span of AD flies, but the effect was more prominent in the AD flies exposed to rivastigmine. This may be due to the reduction in oxidative stress. Oxidative stress results from an imbalance in the production of ROS and anti-oxidative defense system which is responsible for the removal of ROS (Harman, 1981). High levels of protein oxidation, lipids peroxidation, DNA oxidation and the formation of toxic substances such as ketones, aldehyde, peroxides have been implicated not only in brain but also in cerebrospinal fluid, blood and urine of AD patients (Lovell and Markesbery, 2007). The important part of anti-oxidant defense system is GSH which donates electrons to ROS for proper scavenging (Praticò et al., 2000). The use of anti-oxidants has also been reported to reduce the oxidative stress in AD patients as well as in experimental models of AD (Feng and Wang, 2012). GSH, a tripeptide protects cells against oxidation. The antioxidant function of GSH is closely associated with its function in providing reducing environment to the cell with the help of NADPH (Meister, 1995). Glutathione in its reduced form is the most powerful intracellular anti-oxidant. Low levels of GSH indicates the increased level of oxidative stress (Exner et al., 2000). In our present study the AD flies show the low levels of GSH content, but the AD flies exposed to rivastigmine and galantamine showed increased levels of GSH content compared to unexposed AD flies. Rivastigmine was more effective in increasing the levels of reduced GSH compared to Galantamine. GST is involved in the process of detoxification via conjugation of reduced GSH (Nebert and Vasiliou, 2004). It can act on a wide variety of endogenous as well as exogenous substrates (Strange et al., 2001). The levels of GST have been reported to alter in several pathological diseases such as AD, PD and cancer (Kumar et al., 2017). In our study the AD flies showed increased levels of GST activity compared to control flies. The AD flies exposed to rivastigmine and galantamine showed reduction in the activity of GST compared to galantamine. Our earlier studies with the same strain of flies have shown increase in TBARS in brain of AD flies (Beg et al., 2018; Ali et al., 2019). A number of studies have reported an elevated level of malondialdehyde in AD patients (Sinem et al., 2010; Padurariu et al., 2010; Ozcan-kaya and Delibas, 2002; Markesbery et al., 2005). Increased levels of TBARS have also been reported in serum of AD patients (Serra et al., 2009). In our study the exposure of AD flies to rivastigmine and galantamine reduced the levels of TBARS, but the reduction was more significant in AD flies exposed to rivastigmine compared to galantamine. ROS can also oxidize protein and thus increases the levels of protein carbonyl content which is a well-known marker for protein oxidation. AD flies showed marked increase in PC content. Higher content of protein carbonyls has been reported in AD patients (Greilberger et al., 2010). Both rivastigmine and galantamine were effective in reducing the PC content in the brain of AD flies, but rivastigmine was more effective in comparison to galantamine. AD flies showed an increase in the activity of SOD and catalase. The AD flies exposed to rivastigmine showed a dose dependent significant decrease in the activity of SOD on all selected doses. Galantamine significantly reduced the SOD activity at 10 mM and catalase activity of 1 and 10 mM, compared to unexposed AD flies. However, the study on AD patients showed that acetylcholinesterase inhibitors did not influence the activities of catalase and glutathione

reductase, however a significant difference in the activities of catalase and glutathione reductase was observed compared to control group (Klugman et al., 2012). Caspases are the family of protease enzymes which play an essential role in programmed cell death. *Drosophila* genome encodes seven caspases i.e. Dronc, Strica, Dredd, Drice, Dcp-1, decay and Dahh (Hay and Guo, 2006). Drice and Dronc are homologs of mammalian caspases-9 and 3, respectively. In our present study the spectrophotometric detection of the chromophore p-nitroanilide (pNA) obtained after the specific action of caspases-9 and 3 on tetrapeptides substrates acetyl-Asp-Glu-Val-Asp-p-nitroanilide (DEVD-pNA) and AC-Leu-Glu-His-Asp-p-nitroanilide (LEHD-pNA), respectively, was performed (Siddique and Jyoti, 2017). A dose dependent significant decrease in the activity of caspase 3 and 9 was observed in the AD flies exposed rivastigmine and galantamine. The reduction in the activity of caspases-9 and 3 was more in the AD flies exposed to rivastigmine compared to the AD flies exposed to galantamine.

Acetylcholinesterase is found in cholinergic synapses and is responsible for the breakdown for the neurotransmitter acetylcholine. In *Drosophila* most of the activity of acetylcholinesterase is found in the central nervous system. The progressive loss of acetylcholinesterase activity has been reported in AD patients (Shinotoh et al., 2000). The study on cultured retinal cells showed that in the presence of synthetic peptide A β ₂₅₋₃₅, the activity of acetylcholinesterase was increased and the anti-oxidant like α -tocopherol acetate and nitric oxide synthase inhibitors were capable of reducing the activity of acetylcholinesterase (Melo et al., 2003). Acetylcholinesterase inhibitors can influence the processing of amyloid precursor protein (APP) and A β -production thereby responsible for attenuating A β induced toxicity (Nordberg, 2006). It has been suggested that acetylcholinesterase play an important role in A β -aggregation during the early stages of senile plaque formation. Hence, the cholinesterase inhibitors play a dual role i.e. increases the availability of acetylcholine in the brain and decrease the formation of A β -aggregates (Anand and Singh, 2013). Our study demonstrates that both the drugs not only inhibit the acetylcholinesterase, but also reduced the oxidative stress and the aggregation of A β -42. The strategy of designing the drug to target specifically A β process mainly involves blocking, preventing self-assembly, catabolism, removal and counteracting of A β (Walker and Rosen, 2006). Donepezil and rivastigmine has been reported to enhance A β -clearance across the blood brain barrier and liver (Mohamed et al., 2015). In our study the AD flies showed a slight decrease in the activity of acetylcholinesterase compared to control flies. Both the drugs i.e. rivastigmine and galantamine were effective in reducing the activity of acetylcholinesterase, but galantamine was more effective in reducing the activity of acetylcholinesterase, hence the galantamine is more effective inhibitor of acetylcholinesterase compared to rivastigmine. The study on *Dugesia tigrina* for the inhibition of acetylcholinesterase activity, galantamine showed high inhibitory effect compared to donepezil, tacrine and rivastigmine (Bezerra da Silva et al., 2016). Rivastigmine and galantamine are well known acetylcholinesterase inhibitors. The comparative study of these both inhibitors at the same dose was studied on some human AD patients exhibiting features, such as reduced lifespan, locomotor defects and increased oxidative stress (Li et al., 2019; Onor et al., 2007; Takeda et al., 2006).

The neuropil in the insect brain is suggested to be responsible for the coordination on between neuronal information and function (Kahsai and Winther, 2011). A number of neurotransmitters and neuromodulators have been reported in the *Drosophila* brain such as acetylcholine, γ -amino butyric acid (GABA), glutamate, dopamine, serotonin and octopamine (Kahsai and Winther, 2011). From the study of Kahsai and Winther (Kahsai and Winther, 2011) it has been suggested that acetylcholine and glutamate are important primary neurotransmitters of central complex and in combination with neuropeptides play an important role in controlling learning, courtship and locomotor in *Drosophila*. The impairment of glutamate (GABA) glutamine cycle leads to motor deficit and shortens life span (Mazaud et al., 2019). The primary excitatory neurotransmitter in CNS and the sensory neurons in *Drosophila* is acetylcholine (Lee and O'Dowd, 1999), but it is not present in

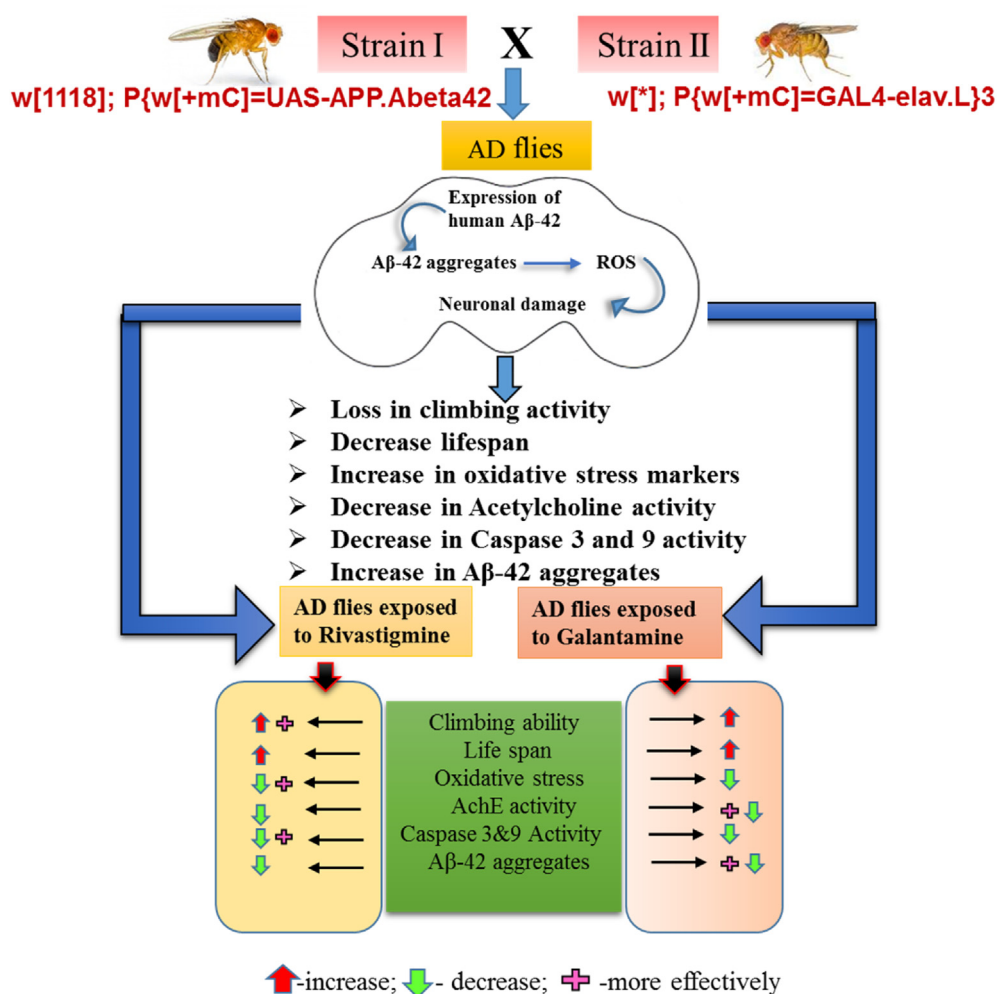


Fig. 6. Schematic representation of the results.

neuromuscular junction as in vertebrate. Despite of having such differences acetylcholine in *Drosophila* regulates jumping, climbing ability and motion (Hou et al., 2003; Takemura et al., 2013).

Acetylcholinesterase inhibitors have been reported to increase the plasma levels of Aβ₄₂ in AD patients (Conti et al., 2010). In brain of AD patients, a reduction in the deposition of Aβ was found who were undergoing with the therapy of cholinesterase inhibitors (Ballard et al., 2007). The results obtained for the Aβ-42 aggregates by performing immunohistochemistry on brain sections also supports the reduction in the formation of Aβ-42 aggregates in the AD flies exposed to rivastigmine and galantamine. The study on human neuroblastoma cell line SH-SY5Y reveals a U-shaped neuroprotective curve for galantamine and donepezil against okadaic acid toxicity (Arias et al., 2005). Maximum protection was achieved at 0.3 μM galantamine, 1 μM of donepezil and 3 μM rivastigmine (Arias et al., 2005). The maximum protection against apoptosis induced by Aβ₂₅₋₃₅ in SH-SY5Y cells was also observed at the same concentrations (Arias et al., 2005). The retrospective comparative analysis of donepezil, rivastigmine and galantamine for the treatment of dementia associated with AD showed donepezil as a more persistent drug compared to rivastigmine and galantamine (Sicras and Rejas-Gutierrez, 2004).

5. Conclusion

It is concluded from the results (Fig. 6) obtained from our present study that rivastigmine exerts the protective effect mainly by reducing the oxidative stress but on the other hand it is also a cholinesterase

inhibitor. Galantamine is more potent cholinesterase inhibitor compared to rivastigmine.

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Ethical statement

Not Required.

Data availability

All data is presented in the manuscript.

CRediT authorship contribution statement

Yasir Hasan Siddique: Participated in research design and Wrote manuscript, Conducted experiments, Performed data analysis. **Falaq Naz:** Conducted experiments, Performed data analysis. **Rahul:** Conducted experiments, Performed data analysis. **Himanshi Varshney:** Conducted experiments.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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