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 now becoming more prevalent in ageing populations worldwide. It 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. 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 Aβ-42 in the neurons. The effect of similar doses of rivastigmine and galantamine (i.e. 0.1,1 and 10mM) was studied on the climbing ability, lifespan, oxidative stress markers, caspase 9 and 3, acetylcholinesterase activity and on the formation of Aβ-42 aggregates. The results suggest that the rivastigmine is more potent in reducing the oxidative stress and improving climbing ability of AD flies. Galantamine is a more potent inhibitor of acetylcholinesterase compared to rivastigmine. Galantamine prevents the formation of Aβ-42 aggregates more effectively compared to rivastigmine. The present study gives a comparative account of the rivastigmine and galantamine on the efficacy. The effect of the drugs taking the same doses have been studied on the climbing ability, life span, oxidative stress markers, apoptotic markers, acetylcholinesterase activity and on the formation of Aβ-42 aggregates. The study has been performed on the transgenic *Drosophila* model of AD expressing human Aβ-42 in the neurons.

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[1]. Currently two types of medication are approved for the treatment of AD. 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[2]. The efficacy of these drugs has been studied in a number of clinical studies[3-7] and in various experimental models[8-12]. Karoger et al[13] 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.[14] 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 are unclear. No clear evidence exists to date whether one of these drugs is more efficacious than another[15]. The comparative study by Aguglia et al[16] 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[17]. Oxidative stress has been reported to be associated with the neuronal death in AD patients[18] and transgenic mice[19]. $A\beta_{1\text{-}42}$ oligomeric species leads to the disorganization of the cytoskeleton thus causing neurite retraction, impaired Ca^{+2} homeostasis, endoplasmic reticulum stress and apoptosis[20-22].

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[23,24]. 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[25]. The newly synthesized acetylcholinesterase inhibitor XJP-1 resulted in a significant improvement of AD symptoms and a reduction of amyloid plaques by reducing the amyloid aggregation take in transgenic *Drosophila* model of AD[25]. XJP-1 also improves the climbing ability and life span of the transgenic flies expressing Aβ-42[25].

There is also a reasonable similarity between the central nervous systems of flies and humans with both consisting of neurons and glia. Both utilize the same neurotransmitters[24]. At present various transgenic *Drosophila* models are available to study the various aspects of neurodegenerative diseases such as Parkinson's Disease, AD and Huntington's Disease[26, 27]. Among these transgenic models one model express human Aβ-42 under GAL4-UAS system in the brain and the flies exhibit diffuse amyloid deposits, age dependent loss in climbing ability, memory, olfaction and neurodegeneration[28-30]. Since, AD is associated with the decline motor activity, memory loss, deposition of Aβ-42 and increase in oxidative stress hence, we decided to study the lifespan, climbing ability and oxidative effect of rivastigmine and galantamine. Immunohistochemistry was also performed on the brain sections to study the effect of rivastigmine and galantamine on the expression of Aβ-42 aggregates.

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 Aβ-42 in the neurons and the flies are referred as AD flies[31].

Drosophila **culture and crosses.** The flies were cultured on standard *Drosophila* food containing agar, corn meal, sugar and yeast at 25°C (24 ± 1)[32]. Crosses were set up as described in our earlier published work[33]. The AD flies were allowed to feed separately on different doses of rivastigmine and galantamine mixed in the diet for 30 days (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[34].

***Drosophila* climbing assay.** The climbing assay was performed as described by Pendleton et al[35].Ten flies were placed in an empty glass vial (10.5 cm× 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.

Estimation of glutathione (GSH) content. The glutathione (GSH) content was estimated colorimetrically using Ellman's reagent (DTNB) according to the procedure described by Jollow et al[36]. The assay mixture consisted of 550 µl of 0.1 M phosphate buffer, 100 µl of supernatant and 100 µl of DTNB. The OD was read at 412 nm and the results were expressed as µ moles of GSH/gram tissue.

Estimation of glutathione-S-transferase (GST) activity. The glutathione-S-transferase activity was determined by the method of Habig et al[37]. The reaction mixture consisted of 500 µl of 0.1M phosphate buffer, 150 µl of 10 mM CDNB, 200 µl of 10 mM reduced glutathione, and 50 µl of supernatant. The OD was taken at 340 nm and the enzyme activity was expressed as μmoles of CDNB conjugates formed/min/mg protein.

Lipid peroxidation assay. Lipid peroxidation was measured according to the method described by Ohkawa et al[38]. 5 µl of 10 µM butylhydroxy toluene (BHT), 200 µl of 0.67% thiobarbituric acid (TBA), 600 µl of 1% orthophosphoric acid (OPA), 105 µL of distilled water and 90 µl of the sample were taken in an eppendorf, vortexed and kept in the water bath at 90°C for 45 minutes. OD was read at 535 nm and the results were expressed in µ moles of TBARS formed/h/gram tissue.

Estimation of protein carbonyl content (PCC). The protein carbonyl content was estimated according to the protocol described by Hawkins et al[39]. The homogenate was diluted to a protein concentration of approx 1 mg/ml. About, 250 µl of diluted homogenate was taken in eppendorf. To it, 250µl of 10 mM 2,4-dinitrophenyl hydrazine (dissolved in 2.5 M HCl) was added, vortexed and kept in dark for 20 minutes. About 125µl 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 minutes 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 1ml of 6M 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[40] by kinetic method where rate of dismutation of H_2O_2 to water and molecular oxygen is proportional to the concentration of catalase in the sample. The reaction mixture consisted of 650 µl of 0.1 M phosphate buffer, 333 µl of H_2O_2 (0.05M) and 17 µl of sample. A decrease in OD was measured for

2 minutes, at 30 sec interval at 240 nm. The activity of catalase was calculated and expressed as µmoles of H_2O_2 consumed/min/mg protein.

Determination of superoxide dismutase activity (SOD) activity. The SOD activity was estimated according to the method of Marklund and Marklund[41]. The reaction mixture consisted of 17 µl of sample and 950 µl of 0.1M phosphate buffer. The reaction was initiated by adding pyrogallol. An increase in OD was noted at 420 nm for 3 minutes at 30 seconds 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 µl of homogenate and 50 µl of chilled cell lysis buffer) was incubated on ice for 10 min. After incubation, 50 µl of 2X reaction buffer (containing 10 mM DTT) with 200 µM substrate (DEVD-pNA for Drice, and IETD-pNA for Dronc) was added and incubated at 37ºC for 1.5 hour. The reaction was quantified at 405 nm.

Acetylcholinesterase (AChE) activity. Acetylcholinesterase activity was determined by the method described by Ellman et al[42]. 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 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 µl of the sample, 650 µl of 0.1 M phosphate buffer and 100 µl of 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB). To the mixture 10 µl of acetylthiocholine was added and the change in OD was recorded at every 1 min interval for 3 mins.

Immunohistochemistry. The fly heads were isolated and the paraffin sections were prepared according to the procedure described by Palladino et al[43]. The sections were deparaffinized and rehydrated. The slides were blocked in 8% Bovine Serum Albumin (BSA) for 2.5 hours. Then the slides were washed with phosphate buffer saline (pH 7.2) containing 2% BSA for 5 minutes. For immunohistochemistry, after washing the slides were incubated with primary antibody (Rabbit monoclonal Aβ42 antibody, Merck; 1:200 dilutions) in a humidified chamber for 12 hours at 4ºC. The slides were then washed with PBS containing 2% BSA for 5 minutes and incubated with secondary antibody (Goat anti-Rabbit IgG, alkaline phosphatase conjugate, Merck, USA) at room temperature for 2 hours. The final wash was given by PBS containing 2% BSA for 5 minutes. 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 Aβ42aggregates were quantified in terms of Aβ-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 ± SEM.

Results

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, in the climbing ability, respectively, compared to AD flies (Fig. 1; $p<0.05$). 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; $p<0.05$).

The results obtained for the life span is shown in Figure 2.The analysis on the 3^{rd}day reveal no significant difference between AD flies, AD flies exposed to rivastigmine as well as galantamine and control flies (Fig. 2; $F=0$; $df=23$; $p<0.05$). The analysis on the 12^{th} day also reveal the similar result (Fig. 2; $F=1$; $df=23$; $p<0.05$). The analysis on the 21^{st} day also reveal no significant difference between AD flies, AD flies exposed to rivastigmine as well as galantamine and control flies (Fig. 2; $F=2.262$; $df=23$; $p<0.05$). The analysis on the 30^{th} 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 (Fig. 2; $F=1.929$; $df=23$; $p<0.05$). 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 39^{th} day reveals a significant difference between AD flies, AD flies exposed to rivastigmine and control flies (Fig. 2; $F=15.937$; $df=23$; $p<0.05$). The AD flies showed a significant decrease of 3.59 folds in the survival rate compared to control flies (Fig. 2; $p<0.05$). 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 (Fig. 2; $p<0.05$). On the 48^{th} day all the unexposed AD flies died compared to control flies and the AD flies exposed 10 mM of rivastigmine (Fig.2; $F=27.974$; $df=23$; $p<0.05$). In all remaining groups the flies were found to be dead on the 57^{th} day (Fig. 2; $p<0.05$).

The AD flies showed a significant decrease of 1.72 fold in the 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 the GSH content compared to unexposed AD flies (Fig. 3a; $p<0.05$). 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 the GSH content compared to unexposed AD flies (Fig. 3a; $p<0.05$). The AD flies exposed to 0.1 mM of rivastigmine and galantamine did not showed significant increase in the GSH content compared to unexposed AD flies (Fig. 3a; $p<0.05$).

The AD flies showed a significant increase of 1.92 fold in the GST activity 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 GST activity 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 GST activity compared to unexposed AD flies (Fig. 3b;

p<0.05). The AD flies exposed to 0.1 mM of galantamine did not showed significant decrease in the GST activity compared to unexposed AD flies (Fig. 3b; p<0.05).

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

The AD flies showed an 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 (Fig. 3d; 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 to unexposed AD flies (Fig. 3d; 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).

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 (Fig. 3e; 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 (Fig. 3e; p<0.05). The AD flies exposed to 0.1 mM and 1mM of galantamine did not showed significant decrease in the activity of catalase compared to unexposed AD flies (Fig. 3e; p<0.05).

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 in the activity of SOD compared to unexposed AD flies (Fig. 3f; 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 (Fig. 3f; 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).

The AD flies showed a significant increase of 3.53 fold in the activity of Caspase-9 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 the activity of Caspase-9 compared to unexposed AD flies (Fig. 4a; 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 the activity of Caspase-9 compared to unexposed AD flies (Fig. 4a; p<0.05). The AD flies exposed to 0.1 mM of galantamine did not showed significant decrease in the activity of caspase-9compared to unexposed AD flies (Fig. 4a; p<0.05).

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

The AD flies showed no significant decrease in the activity of acetylcholinesterase 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 the activity of acetylcholinesterase compared to unexposed AD flies (Fig. 4c; $p<0.05$). The AD flies 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 the activity of acetylcholinesterase compared to unexposed AD flies (Fig. 4c; $p<0.05$).

The results obtained for immunohistochemistry are shown in Fig. 5 (a-i). The AD flies showed a marked age dependent increase in the Aβ-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 Aβ-42 aggregates compared to the unexposed AD flies (Fig. 5i). The AD flies exposed to 0.1, 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 Aβ-42 aggregates compared to the unexposed AD flies (Fig. 5i). The aggregates were quantified by using Image-J software (Fig. 5i).

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 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 Aβ-42 aggregates in a dose dependent manner. Galantamine was found to be more effective in preventing the formation of Aβ-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[44]. 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. The result on various oxidative stress markers reveals that both the drugs are effective in reducing the oxidative stress but the effect was more prominent in the AD flies exposed to rivastigmine. The brain of AD patients has been reported to have excessive oxidative stress, over production of Aβ which leads to the production of Aβ-associated free radical and ultimately cell death[45]. Our earlier studies with the same strain of flies have shown increase in TBARS in brain of AD flies[29,30]. A number of studies have reported an elevated level of malondialdehyde in AD patients[46-49]. Increased levels of thiobarbituric reactive substances have also been reported in serum of AD patients[50]. 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. Reactive oxygen species (ROS) can also oxidized 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[51]. 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[52]. 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[53]. 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[54]. 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[55]. The study on cultured retinal cells showed that in the presence of synthetic peptide $A\beta_{25-35}$ the activity of acetylcholinesterase was increased and the antioxidant like α-tocopherol acetate and nitric oxide synthase inhibitors were capable of reducing the activity of acetylcholinesterase[56]. Acetylcholinesterase inhibitors can influence the processing of amyloid precursor protein (APP) and Aβ-production thereby responsible for attenuating Aβ induced toxicity[57]. It has been suggested that acetylcholinesterase play an important role in Aβ- 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 Aβ- aggregates[58]. Our study demonstrates that both the drugs not only inhibit the acetylcholinesterase but also reduced the oxidative stress and the aggregation of Aβ-42. The strategy of designing the drug to target specifically Aβ process mainly involves blocking, preventing self-assembly, preventing catabolism, removal and counteracting of Aβ[59]. Donepezil and rivastigmine has been reported to enhance Aβ-clearance across the blood brain barrier and liver[12]. 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 in this case the 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[11].

Aduhelm is an immunotherapeutic drug approved by FDA induces the clearance of β-amyloid deposits in the brain[60]. Researchers have targeted Aβ in various biophysical states[61], with anti-Aβ monoclonal antibodies, gantenerumab[62], lecanemab[63] and donamemab[64]. The approval of aducanumab has opened a global debate due to the controversial results[65-66]. Some marine derived compounds such as cytarabine, trabectedin, Eribulin and Ziconotide have been reported to be effective against AD[67]. The quest for the drugs is an ongoing process and requires a rigorous clinical trial for final approval. Rivistigmine 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[6-7].

The neuropil in the insect brain is suggested to be responsible for the coordination on between neuronal information and function[68]. A number of neurotransmitters and neuromodulators have been reported in the *Drosophila* brain such as acetylcholine, Y-amino butytic acid (GABA), glutamate, dopamine, serotonin and octopamine[68]. From the study of Kahasai and Winther[68] 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[69]. The primary excitatory neurotransmitter in CNS and the sensory neurons in *Drosophila* is acetylcholine[70], but it is not present in neuromuscular junction as in vertebrate. Despite of having such differences acetylcholine in *Drosophila* regulates jumping, climbing ability and motion[71-72].

Acetylcholinesterase inhibitors have been reported to increase the plasma levels of $A\beta_{42}$ in AD patients[73]. In brain of AD patients, a reduction in the deposition of Aβ was found who were undergoing with the therapy of cholinesterase inhibitors[74]. 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[75]. Maximum protection was achieved at 0.3μM galantamine, 1μM of donepezil and 3μM rivastigmine[75]. The maximum protection against apoptosis induced by $A\beta_{25-35}$ in SH-SY5Y cells was also observed at the same concentrations[75]. 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[76].

It is concluded from the results 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. For the reduction of oxidative stress, the higher doses of galantamine are effective (Fig. 6).

Declarations

Authorship Contributions

YHS: Participated in research design and Wrote manuscript; YHS, FN, R, HV: Conducted experiments; YHS, FN: Performed data analysis.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Ethical statement

Not Required

Data availability

All data is presented in the manuscript.

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Figures

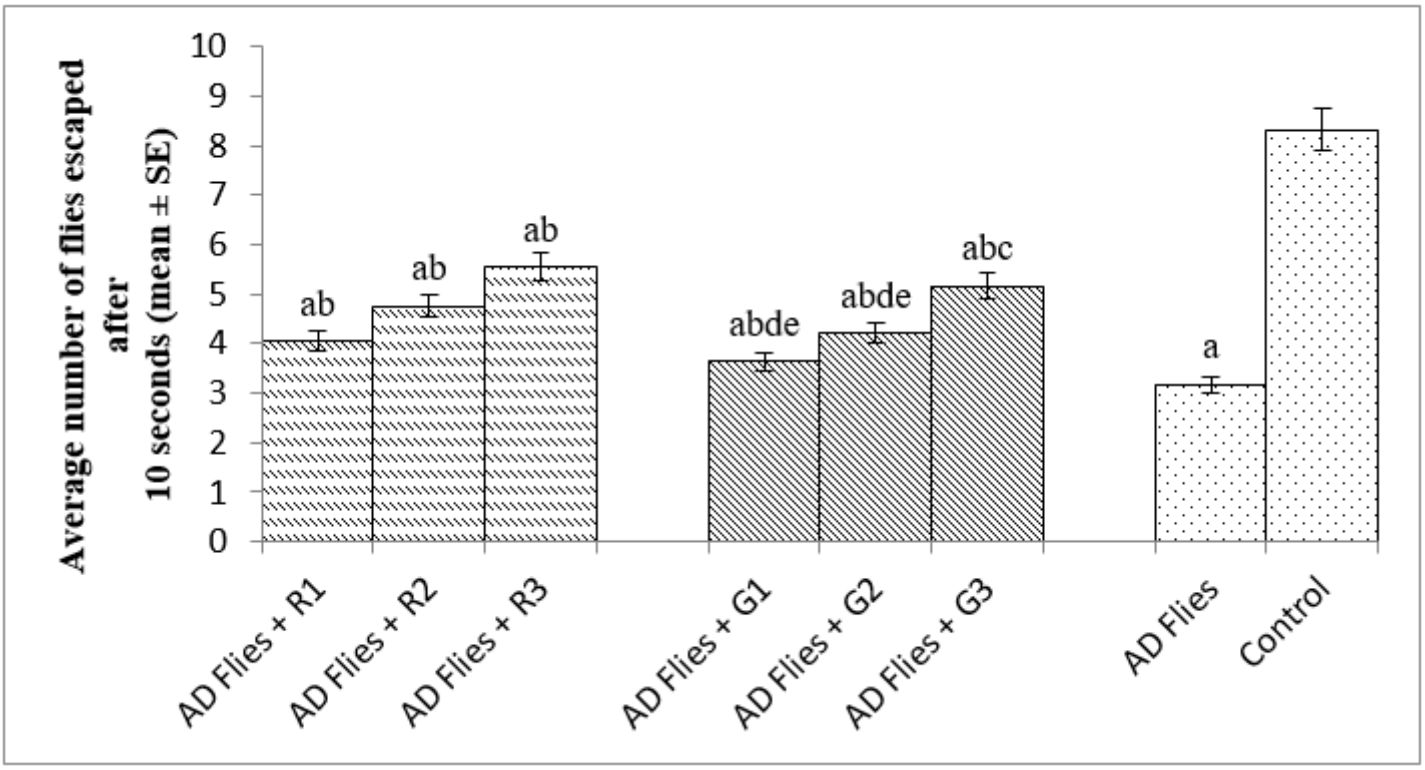


Figure 1

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: Alzhiemer's Disease; R1, G1=0.1, R2, G2=1 R3, G3=10 mM].

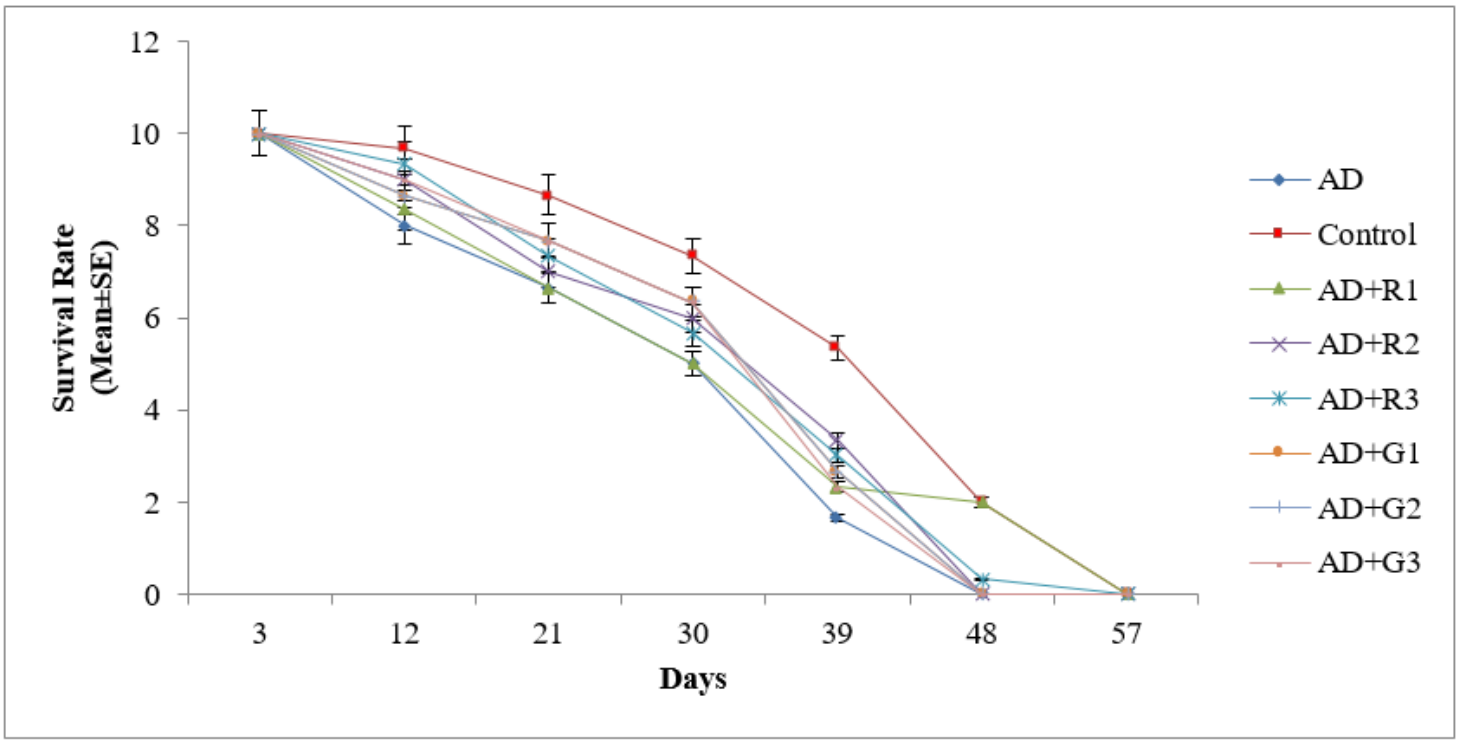
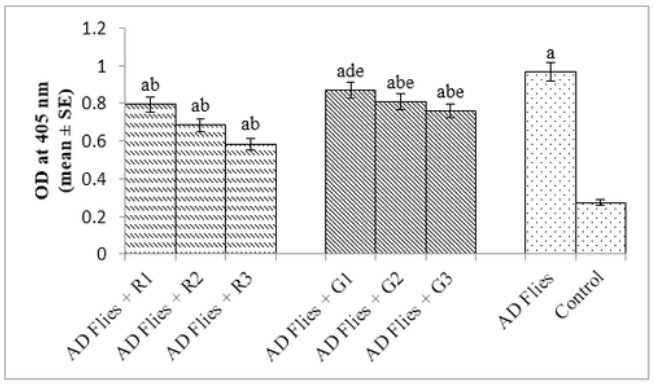


Figure 2

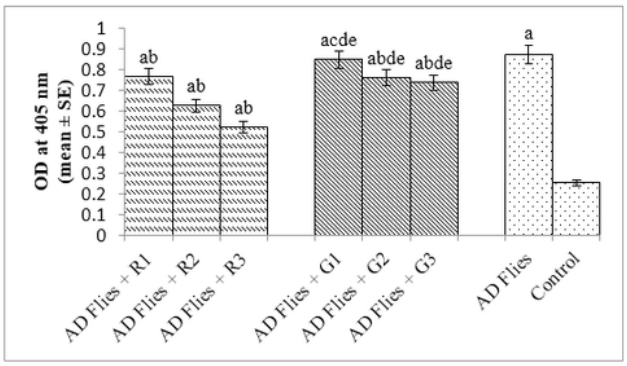
Effect of Rivastigmine (R) and Galantamine (G) on the life span of AD flies. The flies were allowed to feed on the diet supplemented with various doses of R and G till the last fly died. [AD: Alzhiemer's Disease R1, G1=0.1, R2, G2=1 R3, G3=10 mM; {3rd Day (df= 23; F=0), 12th Day (df=23; F=0), 21st Day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

Figure 3

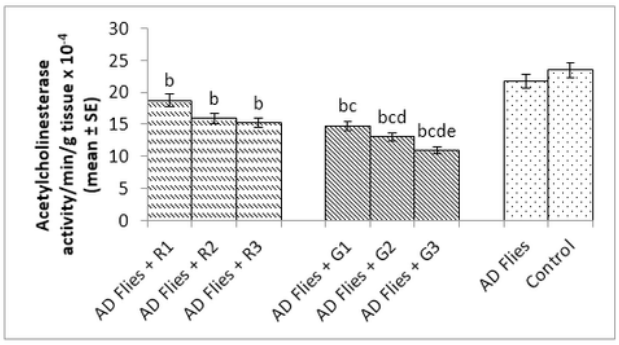
Effect of Rivastigmine (R) and Galantamine (G) on the Glutathione (GSH) (df= 39; F= 62.45)(a), Glutathione-S-Transferase activity (GST) (df=39; F=154.1) (b), TBARS (df=39; F=41.83) (c), Protein Carbonyl content (PC) (df=39; F=88.06) (d), Catalase activity (df=39; F=81.44) (e), Superoxide dismutase activity (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: Alzhiemer's Disease; R1, G1=0.1, R2, G2=1 R3, G3=10 mM].



(a)



(b)



(c)

Figure 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 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: Alzhiemer's Disease;R1, G1=0.1, R2, G2=1 R3, G3=10 mM].

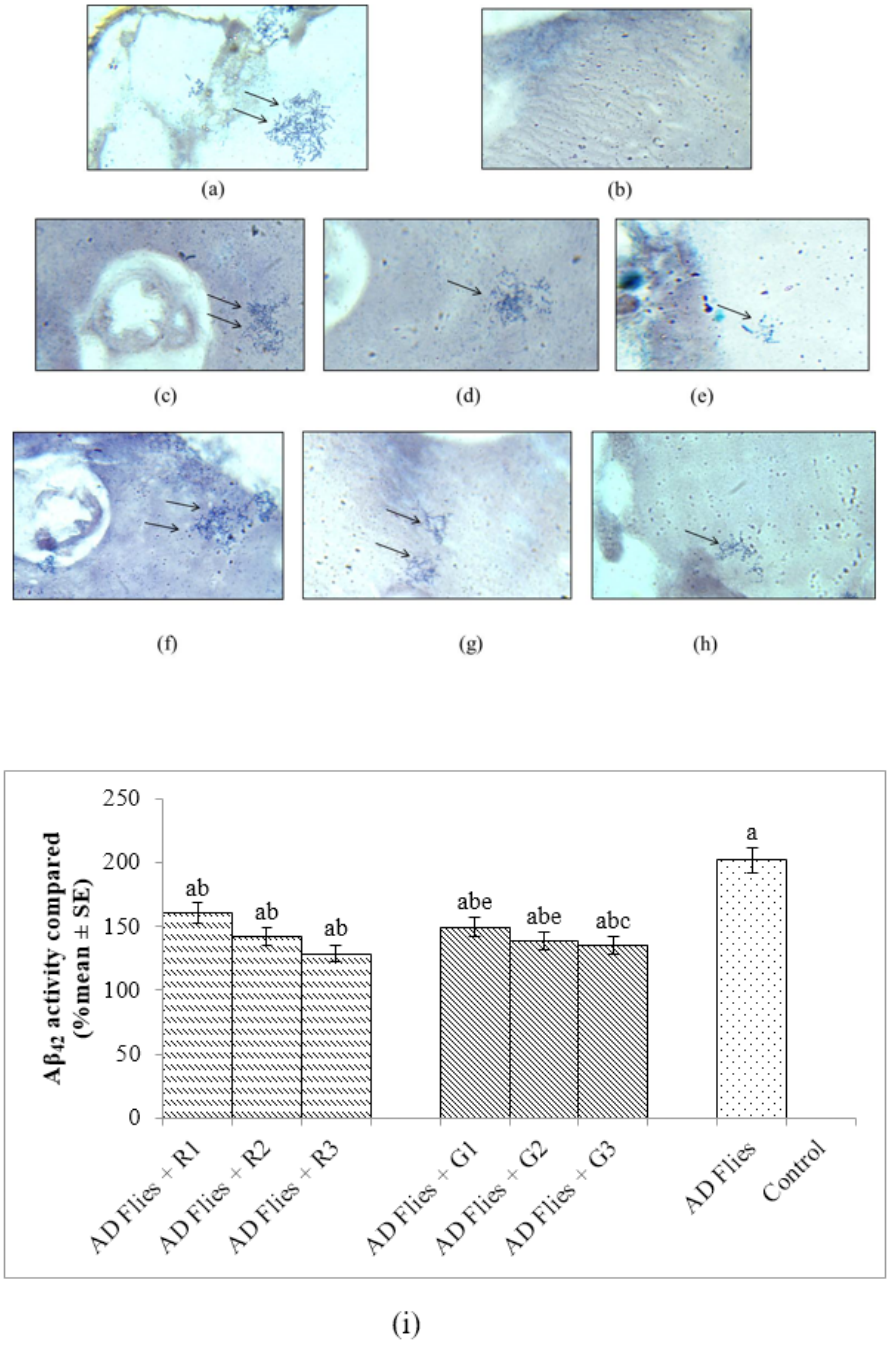


Figure 5

Aβ-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

Aβ-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: Alzhiemer's Disease; R1, G1=0.1, R2, G2=1 R3, G3=10 mM].

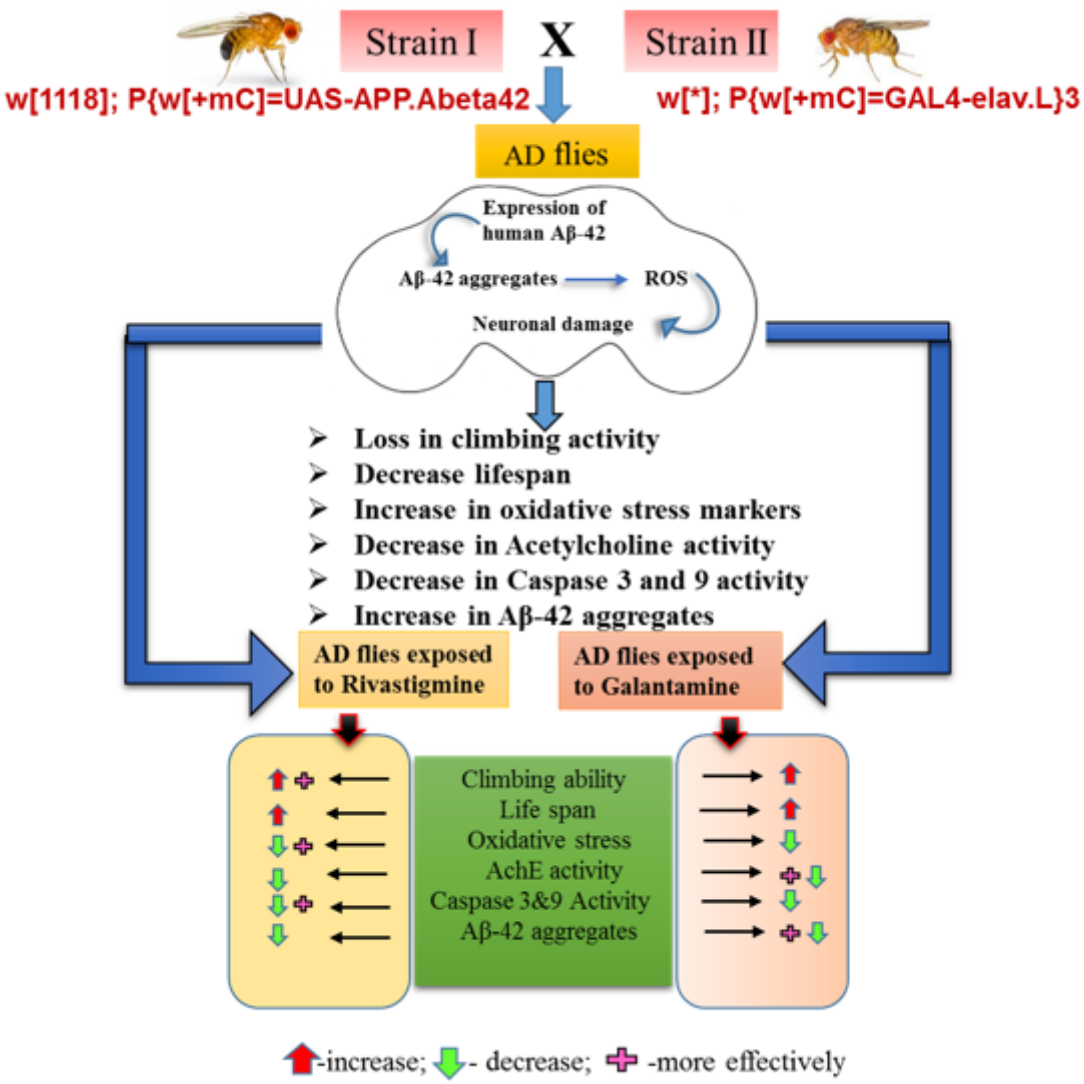


Figure 6

Schematic representation of the results