Novel Therapeutic Mechanism of Action of Metformin and Its Nanoformulation in Alzheimer's Disease and Role of AKT/ERK/GSK Pathway.

Harish Kumar
Post Graduate Institute of Medical Education and Research
Amitava Chakrabarti
Post Graduate Institute of Medical Education and Research
Phulen Sarma
Post Graduate Institute of Medical Education and Research
Manish Modi
Post Graduate Institute of Medical Education and Research
Dibyajyoti Banerjee
Post Graduate Institute of Medical Education and Research
B.D. Radotra
Post Graduate Institute of Medical Education and Research
Alka Bhatia
Post Graduate Institute of Medical Education and Research
Bikash Medhi (drbikashus@yahoo.com)
Post Graduate Institute of Medical Education and Research https://orcid.org/0000-0002-4017-641X

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Abstract

Background: Insulin resistance in brain plays a critical role in the pathogenesis of Alzheimer's disease (AD). Metformin is a blood brain barrier crossing anti-diabetic insulin-sensitizer drug. Current study has evaluated the therapeutic and mechanistic role of conventional as well as solid lipid nanoformulation (SLN) of metformin in intracerebro ventricular (ICV) Aβ (1-42) rat-model of AD.

Methods: SLN-metformin was prepared by the micro-emulsification method and further evaluated by zetasizer and scanning electron-microscopy. In the animal experimental phase, AD was induced by bilateral ICV injection of Aβ using stereotaxic technique, whereas control group (sham) received ICV-NS. 14 days post-model induction, ICV- Aβ treated rats were further divided into 5 groups: disease control (no treatment), Metformin dose of (50mg/kg, 100mg/kg and 150 mg/kg), SLN of metformin 50mg/kg and memantine 1.8mg/kg (positive-control). Animals were tested for cognitive performance (in EPM, MWM) after 21 days of therapy, and then sacrificed. Brain homogenate was evaluated using ELISA for (Aβ (1-42), hyperphosphorylated tau, pAKTser473, GSK-3β, p-ERK,) and HPLC (metformin level). Brain histopathology was used to evaluate neuronal injury score (H&E) and Bcl2 and BAX (IHC).

Results: The average size of SLN-metformin was <200 nm and was of spherical in shape with 94.08% entrapment efficiency. Compared to sham, the disease-control group showed significantly higher ($p \leq 0.05$) memory impairment (in MWM and EPM), higher hyperphosphorylated tau, Aβ (1-42), and Bax and lower Bcl-2 expression. Metformin was detectable in brain. Treatment with metformin and its SLN form significantly decreased the memory impairment as well as decreased the expression of hyperphosphorylated tau, Aβ(1-42), Bax expression and increased expression of Bcl-2 in brain. AKT-ERK-GSK3β-Hyperphosphorylated tau pathway can be implicated in the protective efficacy of metformin.
Conclusion: Both metformin and SLN metformin is found to be effective as therapeutic agent in ICV-AB rat model of AD. AKT-ERK-GSK3β-Hyperphosphorylated tau pathway is found to be involved in the protective efficacy of metformin.

Background

Alzheimer's disease (AD)-related dementia is one of most prominent types of dementia, particularly among the elderly.(1) As the older age population is increasing, the burden of AD is also increasing.(2, 3) Pathophysiologically, AD is characterized occurrence of neuritic plaque (amyloid-beta plaque) and neurofibrillary tangles (owing to hyperphosphorylated tau) in brain(4, 5), neuronal inflammation, synaptic loss, neuronal death and brain dystrophy(5).

There is a strong connection is observed between brain insulin resistance and occurrence of AD (6). Impaired insulin signaling and subsequent brain energy deprivation is seen in the brain of AD (7). Synaptic dysfunction, neuro-inflammation, and autophagic impairments are sharing features of both AD and type-2 DM, which indirectly affect both Aβ and tau functions of neurons (8). AD is sometimes termed as type 3 diabetes (i.e. Brain Diabetes Mellitus).(9–11)

Metformin is an oral anti-diabetic drug, which crosses blood brain barrier (12–16) and also accumulates in CNS(16). Metformin reduces the peripheral and central mitochondrial oxidative stress, redecorates mitochondrial function, prevents depolarization of mitochondrial membrane, and neuro-inflammation(17). Metformin is also found to improve cognitive and executive functions(14), neuroprotection(18) and increased neurogenesis(19). In clinical studies, diabetic people on metformin have better cognitive function than the people on other anti-diabetics(18).

Many studies evaluated the “preventive effect” of metformin in AD. However, we can’t predict occurrence of AD before-hand and there is no highly sensitive and specific test available to detect AD in early prodormal stage, hence a preventive therapy may be less helpful. We investigated the therapeutic efficacy of metformin in this study (not prophylactic). Metformin's exact mechanism of action in AD (18) and other neurodegenerative illnesses is still unknown.(20). Although the ERK/AKT/GSK pathway is implicated in insulin signalling(21, 22)and this pathway is explored with regards to metformin efficacy in other disease conditions e.g. thyroid cancer (23) nonalcoholic steatohepatoitis and cirrhosis (24) etc, but this pathway is not explored with regards to metformin efficacy in AD.

Solid lipid nanoformulation (SLN) has the potential to revamp penetrability of a drug across the blood-brain barrier(25). In case of diabetes mellitus, SLN formulation of metformin could increase the therapeutic efficacy as compared to conventional form, even in smaller dose.(26) Although the permeability of other metformin nanoformulation in brain is evaluated in previous studies, SLN is not evaluated till now. In the present study, we additionally evaluated therapeutic efficacy of solid lipid nanoformulation of metformin in rodent models of AD.

This study, was conducted in insulin plenty environment to evaluate the therapeutic role of metformin. Again metformin role on AKT-ERK-GSK3β pathway remains unexplored. In this study, we have evaluated the role of AKT-ERK-GSK3β pathway in the neuroprotection of AD. Again we are evaluating the therapeutic efficacy of SLN metformin for the first time in case of AD.

Materials & Methods

Experimental animals:

The study was conducted on male wistar rats obtained from the small animal research facility of the institute. Animals were kept in a temperature-controlled environment (25°C) with a 12-hour light/dark cycle and had free access to food and water. One week before to the start of the trial, the animals were acclimatized. The study protocol was approved by Institutional Animal Ethics Committee (Ref. No. 86/IAEC/581). The details of experimental timelines is showed in **Figure 1.**

Grouping:-

Group-I: Sham (n=12)

Group-II: Aβ (ICV)(n=12)

Group-III: Aβ (ICV) + memantine (1.8mg/kg)(n=12)

Group-IV: Metformin 50mg/kg (oral) + Aβ (ICV) (n=12)

Group-V: Metformin 100mg/kg (oral) + Aβ (ICV)(n=12)

Group-VI: Metformin 150mg/kg (oral) + Aβ (ICV)(n=12)

Group-VII: Metformin 50 mg/kg (SLN) + Aβ (ICV). (n=12)

Drugs & chemicals:

Metformin Hydrochloride (extra pure) was obtained from the "research-lab fine chem Industries Mumbai (Cat no. 0999B 00100)". Aβ (1-42) peptide was obtained from GenScript® (cat no. RP10017)". Xylazine® injection U.S.P by Indian immunological Ltd purchased from the market and ketamine HCL injection (Jackson Laboratory Pvt. Ltd®) obtained from hospital supply. Metformin solid lipid nanoformulation was prepared in-house.

Metformin brain level estimation by HPLC:

HPLC (LC-20AD, Shimadzu Corporation Kyoto Japan) was used to evaluate the level of metformin to calculate entrapment efficacy of nanoformulation and evaluation of brain level of metformin. We estimated the level of metformin in brain using reverse phase chromatography using C18 column (phenomenex) and the mobile phase comprised of acetonitrile and phosphate buffer at a ratio of 65:35 [pH =5.75, adjusted with o-phosphoric acid, filtered through 0.2 µm filter], injection volume 50 µl, flow rate 1ml/min and detected at wavelength of 233.0 nm] (27). The mean retention time of metformin nearly found was 1.4 min.

SLN preparation and evaluation details:

SLN was prepared by the micro-emulsification method using aqueous Phase (Double Distilled Water), Surfactant (Pluronic F-127), Lipid (Compritol) and drug (Metformin Hcl). Details of SLN preparation is showed in **Figure 2.** Particle size determination and poly-dispersity index was done by the zetasizer. Reading was taken thrice and the mean value was reported as the final reading. Morphological assessment of the nanoformulation was done by scanning electron microscope (JEOL, JSM-IT300LV JAPAN). The diluted nanoformulation samples were air dried on an aluminum stub and then platinum coating was applied with auto fine coater (JEOL JEC-3000FC) and then the examined under the scanning electron microscope for morphology and images was captured at 20000x (28).

Entrapment efficacy: The formulation was centrifuged at 40000 rpm for one hour on 4^{o}C. Clear supernatant was taken and diluted appropriately (10000 times) and examined under UV spectrometer wavelength 232nm and entrapment efficiency determined using the following equation.(28, 29)

Entrapment efficiency= [(Total drug drug content)−(free drug content)] * 100÷ (total drug content).

Alzheimers disease model induction: Intra-cerebro-ventricular (ICV) Aβ model:

ICV injections were given as per procedure mentioned by Ishrat T et al. (30). Briefly, after being anaesthesized with inraperitoneal (i.p.) ketamine (100mg/kg) and xylazine (5mg/kg), the animals were fixed in the stereotaxic apparatus. Skull was exposed and position of bregma was located. The stereotaxic coordinates used for locating lateral ventricles with relative to bregma were -0.8mm (antero-posterior), 1.5 mm (lateral) and -0.4mm (dorso-ventral). Holes were made bilaterally with the help of drill and ICV injections were administered using a Hamilton® syringe of 10µl.

The sham group received normal saline ICV bilaterally. Animals in the other groups received amyloid β (1-42) peptide 2 µl in distilled water (5µg/µl, incubated at 37^{o}C for one week) bilaterally.(31)

Blood glucose level: Blood glucose level (tail tip) was estimated with the help of portable glucometer as per instructions provided with the device.

Evaluation of cognitive performance: Morris Water Maze:

Spatial memory of the rodents were evaluated with Morris water maze (32-34). All the rats were subjected to four day training with platform and on 5th day probe trial was conducted without platform in which rat were allowed to swim freely for a 90 seconds (cutoff time).

Data extracted were in the form of “escape latencies to find the platform” (during training) and “total time in the platform quadrant” (in probe trial).

Elevated Plus Maze test (Retentive memory):-

Retentive memory was evaluated with elevated plus maze [33, 35]. Training was given for two days (maximum 90 sec) followed by final evaluation. Transfer latency was calculated, which is the time taken to move from open arm to close arm by the rats.

Preparation of brain homogenate: Brain was extracted and kept in PBS (pH 7.4) at -80^{o}C. The brain was then homogenized with PBS and homogenate was subsequently centrifuged at 2000- 3000 RPM for twenty minutes and supernatant was collected carefully to use in further assays.

Estimation of pAKT ser473 level, GSK-3β, p-ERK level, Aβ (1-42) and hyperphosphorylated tau level:

Double antibody sandwich ELISA was used for evaluation of the level of pAKT ser473 level, GSK-3β, p-ERK level, Aβ(1-42) and hyperphosphorylated tau in brain homogenate and were used as per manufacturer instructions. LISA plus microplate reader® was used (wavelength 450 nm in all cases).

Histopathology: Neuronal injury score:

Rats were *anaesthetized* and cardiac perfusion was done using first with normal saline followed by freshly prepared 4% phosphate-buffered para-formaldehyde having (pH 7.4). The brains were harvested,

stored to fix in 4% para-formaldehyde, paraffin embedding of tissue was done to get the desired slice section of the brain on the slide by microtome. Sections thickness 4 µm were harvested from for hippocampal and cortex on the specially treated slide for histopathology and IHC procedure.

Brain hispathology slides (hippocampus and cortex region) were prepared and were stained using hematoxylin and eosin (H&E) staining as mentioned by Myung RJ et al.(36) Histopathological neuronal injury scores was used to evaluate the number of injured and apoptotic neurons in hippocampal and cortex part of the brain. No evidence of neuronal injury to rare occasional apoptotic neuron was given a score of 0; <5 clusters (rare)=1; 5 to 15 clusters of apoptotic neuron=2; >15 clusters=3 and diffuse neuron injury=4. (36)

Bax and Bcl-2 by Immunohistochemistry:

Immunohistochemistry procedure was used as described previously by Zhang TJ et al.(37) Primary antibody used were against Bax (cat.No.Sc7480) and Bcl-2 (cat.No. Sc7382) which were purchased from the Santa Cruz Biotechnology, Santa Cruz, CA and stored at 4°C. DAB was used as chromogen. DAB staining was followed by counterstaining with haematoxylin. Then the tissue was dehydrated with different rising concentration of ethanol followed by air drying and then slides were fixed for observation.

Statistical analysis:

Data were represented as mean ± SD or and median, inter quartile range (IQR) depending upon distribution of data. For categorical versus continuous data, appropriate statistical test was used depending upon the distribution of continuous data. For data showing Gaussian distribution, parametric tests were applied (independent t-test, paired t-test, one-way ANOVA and repeated-measure ANOVA). For data not showing normal distribution, non parametric test was used (Kruskal Wallis test) or other appropriate non-parametric counterpart test was applied as appropriate. Appropriate post hoc test was applied for intergroup comparisons. Value at a level of $p<0.05$ was considered significant. Data analysis was carried out using SPSS.

Results:

Characterization of the solid lipid nanoformulation:

Standard curve of the metformin was prepared with the increasing concentration (0.1, 0.2, 0.3, 0.4. µg/ml) and absorbance was measured and standard curve was plotted, with which various concentration of the unknown sample was measured. Entrapment (loading) efficiency of the metformin in the solid lipid nanoformulation was found to be 94.08%. When evaluated by scanning electron microscopy (magnification x20000), the particles showed spherical shape, and size (<200nm in diameter) of the SLN nano-particles. Data showed in **Figure 3.**

Standardization of Aβ induced dementia model by MWM and EPM:

In MWM, on day 14th post model induction, a higher “latency time to reach the platform” was seen among the animals in the ICV Aβ treated group compared to the ICV normal saline treated animals. In EPM, on day 14th post-model induction, ICV Aβ treated animals showed higher “latency time to reach close arm” compared to the ICV-normal saline treated animals at same time-point and compared to same group baseline data. **Data showed in Figure 4 & 5.**

Cognitive performance in MWM:

Data is shown in **figure 4.** At baseline no significant difference was found in terms of “latency time to reach the platform” between sham and all other groups at same time point in MWM.

At 14 days post model induction, all the assigned groups (Aβ, Memantine, Metformin 50, 100 and 150 mg/kg and SLN-Metformin 50 mg/kg) showed significantly higher latency time to reach the platform when compared to the respective sham group at that same time point.

At 14 days post model induction, except sham all the other assigned groups (Aβ, Memantine, Metformin 50, 100 and 150 mg/kg and SLN- Metformin 50mg/kg) showed significantly higher “latency time to reach the platform” when compared to the respective baseline data of the same group.

When comparing the disease control (Aβ) group to the sham group at 21 days, the disease control (Aβ) group had a significantly longer “latency time to reach the platform” than the sham group at the same time. At 21 days, all other treatment groups (Memantine, Metformin50, 100, and 150mg/kg, and SLN-Metformin50mg/kg) took significantly smaller time to reach the platform than the disease control group. At 21 day post treatment, all treatment groups (Memantine, Metformin 50, 100 and 150mg/kg, and SLN-Metformin 50mg/kg) showed significantly lower “latency time to reach the platform” when compared to the 14 day post model induction data of the same respective group. At 21 days post treatment, no significant different between different treatment groups (Metformin50mg/kg, Metformin100mg/kg, Metformin 150mg/kg, SLN- Metformin50mg/kg) when compared to the positive control group (memantine) at same time point.

Elevated Plus Maze (EPM) Aβ model:

Data is shown in **figure 4.** At baseline no significant difference was seen in terms of “latency time to reach close arm” between sham and all other groups at same time point in EPM.

At 14 days post model induction, all the assigned groups (Aβ, Memantine, Metformin 50, 100 and 150mg/kg, and SLN-Metformin50mg/kg) showed significantly higher latency time to reach close arm when compared to the respective sham group at that same time point. At 14 days post model induction, except sham all the other assigned groups (Aβ, Memantine, Metformin 50, 100 and 150mg/kg and SLN-Metformin50mg/kg) showed significantly higher latency time to reach close arm when compared to the respective baseline data of the same group.

At 21 days, the disease control (Aβ) group showed higher latency time to reach close arm when comparison to the sham group at the similar time point. Again, compared to the twenty-one days disease control group, all other treatment groups (Memantine, Metformin50mg/kg, Metformin100mg/kg, Metformin 150mg/kg, and SLN-Metformin50mg/kg) showed significantly less latency time to reach close arm at same time point. At twenty-one day post treatment, all treatment groups (Memantine, Metformin50mg/kg, Metformin100mg/kg, Metformin 150mg/kg, and SLN- Metformin50mg/kg) showed significantly lower latency time to reach close arm when compared to the 14 day post model induction data of the same respective group. At twenty-one days post treatment, there was no significant different between different treatment groups (Metformin50mg/kg, Metformin100mg/kg, Metformin 150mg/kg, SLN- Metformin50mg/kg) when compared to the positive control group (memantine) at same time point.

Molecular parameters in brain homogenate:

Aβ (1-42) level in brain homogenate in Aβ model: Aβ (1-42) level in brain was significantly higher ($p<0.05$) in disease control as compare to sham.

However, following treatment with Memantine, Metformin 50, 100 and 150 mg/kg and SLN-Met 50mg/kg, a significant decrease in Aβ (1-42) level was observed when compared with disease control group. **Figure-6(A).**

Hyperphosphorylated Tau Level in Aβ Model:

Data is shown in **figure-6(B).**

Hyperphosphorylated-tau level in brain was significantly higher in disease control as compare to sham. Compared to disease control (Aβ) group, hyperphosphorylated tau level was significantly lower in memantine, Metformin (50, 100 & 150 mg/kg) and SLN-Met-50mg/kg group. There was no significant difference among the standard (memantine), different doses of Metformin (50, 100 &150 mg/kg) and SLN-Met-150mg/kg group with regards to hyperphosphorylated tau level.

pAKTser473 level in brain of Aβ induced model:

pAKTser473 level level in brain was significantly lower ($p<0.05$) in disease control as compare to sham. Higher pAKT-ser473 level observed in memantine, metformin 50, 100 and 150mg/kg treated group as compared to disease control,. No significant difference was observed between memantine and different doses of metformin (50, 100 & 150mg/kg).

Although, SLN-Met-50mg/kg group showed a high pAKT ser473 level when compared to disease control (Aβ), but the difference was not statistically significant. Again in pairwise comparison, no significant difference was seen in pAKT ser473 level between the SLN-Met-50mg/kg, sham group, memantine and different doses of metformin (50, 100 & 150 mg/kg). **Figure-6(C).**

p-ERK level in brain in Aβ model:

The Aβ (Disease control) group showed significant decrease in the level of the p-ERK level when compared to sham group. But all other treatment group does not showed any significant difference in p-ERK level when compared to the sham group. Treatment with Memantine and Met150mg/kg significantly increase the level of p-ERK as compared to disease control. But Met50mg/kg, Met100mg/kg and SLN-Met-50mg/kg does not showed any significant difference when compared to Aβ model group. On comparing the Met50mg/kg, Met100mg/kg, Met150mg/kg and SLN-Met-50mg/kg with memantine and sham group does not showed any significant difference. **Figure-6(D).**

GSK3β level in Aβ induced model: GSK3β level significantly increase in disease control compared to the sham. GSK3β level was significantly lower in memantine, met 50mg/kg, met100 mg/kg, met150 mg/kg and SLN-Met50mg/kg group as Compared to disease control. There was no significant difference among the standard (memantine), different doses of Metformin (50, 100 & 150 mg/kg) and SLN-Met50mg/kg group with regards to GSK3β level. **Figure-6(E).**

Metformin concentration in brain:

Metformin was detectable in brain **Figure-7**. Although brain level of metformin increased as the dose of metformin increased in a dose dependent manner, it was not statistically significant.

Although the mean brain level of metformin in SLN-Met50 was higher than conventional metformin 50 mg/kg , but not statistically significant. No significant difference was seen between SLN-Met 50 and different doses of metformin (Met-50mg/kg, Met-100mg/kg, Met-150mg/kg). **Figure-7.**

Blood glucose level in Aβ induced model:

There was no significant increase in blood glucose level after Aβ-ICV injection as mentioned in **figure-8**.

Histopathological findings: H&E staining:

Hippocampus: Normal hippocampal histopathology was seen in the sham group, higher level of pyknotic neurons were seen in the Aβ group. All other treatment groups' i.e. Memantine, Met50mg/kg, Met100mg/kg, Met150mg/kg and SLN-Met50mg/kg groups in hippocampus showed lesser neuronal injury as compared to to Aβ model group. **Figure-9.**

Cortex: Sham group in cortex showing normal neuron. But neuronal injury increased in disease control group as compare to the sham. All other treatment groups i.e. Memantine, Met50mg/kg, Met100mg/kg, Met150mg/kg and SLN-Met50mg/kg in cortex showed lesser neuronal injury as compared to the Aβ model group in cortex. **Figure-9.**

Neuronal injury score (H&E) in Aβ (1-42) induced model:

Data shown in **figure-9(H)**. Neuronal injury score was significantly higher in disease control as compare to sham. But neuronal injury score was not significantly different in Memantine, metformin (50 ,100, & 150 mg/kg) and SLN-Met 50 group as compared to disease control. But at the same time the neuronal injury

score of the Memantine, Metformin (50 , 100 &150 mg/kg) and SLN-Met 50 group were not significantly different as compared to the sham group either.

Immunohistochemistry for Bcl-2 in brain:

Result of BCL2:

Hippocampus: Hippocampus in sham group showing the normal Bcl2 expression. In the disease control group, Bcl2 expression was lower than in the sham group. All other groups' i.e. Memantine, Met50mg/kg, Met100mg/kg, Met150mg/kg and SLN-Met50mg/kg groups showed higher Bcl2 expression when compared to to Aβ model group (disease control). **Figure-10**

Cortex: Sham group in cortex showing normal Bcl2 expression. But Bcl2 expression was lower in the disease control group as compare to the sham. All other groups i.e Memantine, Met50mg/kg, Met100mg/kg, Met150mg/kg and SLN-Met50mg/kg showed higher level of Bcl2 expression as compared to the Aβmodel group. **Figure-10.**

Bax expression in Aβ model:

Result of BAX

Hippocampus: Hippocampus in sham group showing the normal Bax expression but in Aβ group, Bax expression increased when compared to the sham. All other groups i.e. Memantine, Met50mg/kg, Met100mg/kg, Met150mg/kg and SLN-Met50mg/kg groups showed decrease in Bax expression when compared to to Aβ model group. **Figure-11.**

Cortex: Sham group in cortex showing normal Bax expression. Expression of Bax increased in disease control group as compared to the sham. All other groups i.e. Memantine, Met50mg/kg, Met100mg/kg, Met150mg/kg and SLN-Met50mg/kg in cortex showed decreased Bax expression as compared to the Aβ model group. **Figure-11.**

Discussion:

In present study, we explored therapeutic potential of metformin and SLN metformin in insulin plenty environment and the role of AKT-ERK-GSK3β pathway in metformin mediated neuroprotection in AD.

SLN of metformin:

In our study we have prepared the metformin SLN by micro-emulsification method. Because of lipophilic nature, SLN can easily cross the blood brain barrier. (38) Even SLN can also release drug sustainably for longer duration so it can improve the patient compliance and produce action for longer duration. Compritol-based (lipid base) SLN is best carrier for brain drug delivery. Compritol also increases the drug entrapment efficiency in the nanoformulation.(39)

In present study, the entrapment efficiency of metformin SLN was found to be 94.08%. In morphological analysis with the help of scanning electron microscopy (SEM) nanoparticles of SLN was found to be mostly in spherical shape**.** In this SLN Particle size was less than 200 nm as shown in the SEM. SLN having particle size less than 200 nm have good entrapment efficiency as well as it can readily cross the BBB for treatment of Alzheimer's disease. (40) (41) SLNs readily captures in brain because of its lipid base which is a favorable condition to cross BBB.(40)

Metformin brain permeability and effect of SLN formulation:

Regarding brain level of metformin, in present study**,** it has been found that after 21 days treatment, both conventional metformin and SLN metformin level was detectable in brain which indicate BBB permeability of both the formulation (conventional metformin and SLN metformin). Compared to conventional formulation although the permeability of nanoformulation seemed higher when compared to the metformin 50mg/kg, but the difference was not statistically significant. Similar BBB crossing by metformin and its CNS effects are also reported by previous studies(12-16, 42, 43).

Standardization of I.C.V. Aβ model of AD:

ICV-Aβ injection in rodent is a well validated model of AD with high construct, face and predictive validity. (44)

Intra-cerebroventricular (ICV) injection of Aβ1-42 in rat brain gets diffused into entire brain and after seven days of single injection animals start showing memory loss and decrement in hippocampal neuronal plasticity(45). The sequelae of ICV-Aβ injections in rodent closely mimic the Alzheimer's like condition(46).

We have established and standardized the i.c.v. Aβ model in our lab conditions and while standardizing, we have used a battery of memory parameters which included elevated plus maze (EPM) and morris water maze (MWM). After 14 days of ICV injection, the disease group showed significant increase in both latency time to reach the platform in MWM (signifies loss of spatial memory) and latency time to reach the close arm in case of EPM (signifies loss of retentive memory) when compared to the sham group and compared to baseline value of the same group. This confirms a dementia like state in the disease control groups. Similar finding was seen in study by Kasza Á et al (45, 47) For standardization of the model, we confirmed neuronal damage by histopathology and later evaluated pro-apoptotic (Bax) and anti-apoptotic (Bcl2) factors by IHC. Animals in which AD was induced (by using ICV AB) showed enhanced neuronal damage, expression of pro-apoptotic factors increased and anti-apoptotic factors decreased when compared to sham. All these findings confirm that, the ICV injection of Aβ1-42 induced a state of neurodegeneration and dementia in our experimental animals. However, ICV injection of AB did not alter the peripheral blood glucose level.

Evaluation of therapeutic efficacy of metformin:

In present study, treatment with metformin post model induction for 21 days was associated with improvement in spatial (MWM) and retentive memory (EPM), however, no significant difference was seen between different doses of metformin. Similar findings are also reported earlier(14). Brain Aβ level is a surrogate marker of Alzheimer's disease. In the present study, model induction was confirmed by presence of higher Aβ1–42 level in disease control group. Treatment with Metformin was associated with lower Aβ1–42 level in brain of Aβ(1-42) induced model of Alzheimer like dementia. Present study finding are also supported by Chen et al., which states that metformin significantly reduced neurotoxic Aβ1–42 level in hippocampus and reduced apoptosis and reduced memory impairment in db/db mice.(48) The AB1-42 lowering action of metformin is supported by findings from other studies (48).(49) (50).

The disease control group of our study showed higher hyper-phosphorylated tau level in the brain. Treatment with metformin decreased its level however, no dose dependent effect was seen. Metformin protective action can be attributed to its action on phosphosites and it is reported that metformin causes dephosphorylation of tau at the site responsible for the phosphorylation in AD. So it can have a disease modifying effect in reversing AD pathology.(51, 52) Similar to our study, Li et al., demonstrated that treatment with Metformin attenuated the increase in total tau and phosphorylated tau which are hallmarks of AD pathogenesis.(53) In primary neuronal culture obtained from tau transgenic mouse also, treatment with metformin decreased tau phosphorylation indicating its possible use as a disease modifying agent.(54) In a P301S tauopathy model also, metformin observed to decrease tau phosphorylation.(55, 56)

Efficacy of SLN metformin:

In our study, treatment with SLN-Met50mg/kg resulted in significant improvement in spatial memory (MWM) and retentive memory (EPM). But there was no significant difference in memory when SLN was compared with different doses of metformin and positive control. When SLN-Met50mg/kg was compared with met 50mg/kg, although the latency time (in EPM) was less in the SLN-Met50mg/kg group, the difference was not statistically significant.

Treatment with SLN-Met50mg/kg led to decrease in the level of Aβ (1-42) in brain when compared to disease control group. But in Aβ (1-42) model, this decrease was not significantly different from different doses of metformin and positive control. There was no difference in effect of SLN-Met50mg/kg and positive control on Aβ (1-42) level.

In this study with regards to level of hyperphosphorylated tau level in brain, treatment with metformin SLN-Met50mg/kg resulted in significantly lower hyperphosphorylated tau level when compared to disease control in Aβ (1-42) models. But there was no significant difference between different doses of metformin (50, 100 & 150mg/kg), positive control and SLN-Met50mg/kg.

To summarize, treatment with SLN-Met50 mg/kg improved the memory parameters (spatial and retentive memory), decreased Aβ (1-42) and hyperphosphorylated tau level when compared to the disease control group. However, the difference between met-50 mg/kg and SLN-Met50 mg/kg is not significant in case of

memory parameters and hyperphosphorylated tau level. There was no significant difference between SLN-Met50mg/kg and positive control with regards to any of the efficacy parameters. Size of nanoparticles in present study is supported by Dhawan S et al., in which they shows that the compritol based SLN having particle size less than 200 nm show good entrapment efficiency and can readily cross the blood brain barrier for treatment of Alzheimer's disease.(41)

Many studies report increase in amyloid beta level following metformin therapy. Chen Y et al., found that metformin when given alone, increased Aβ level. But in presence of insulin, it enhanced the amyloid beta clearing action of insulin.(13) In our study, metformin decreased amyloid beta level. Again, the blood glucose level of all the animals used in our study, were within normal range, which indirectly highlights the presence of adequate amount of insulin. Thus lowering of amyloid beta level following metformin treatment in our study is in accordance with Chen Y et al., these findings highlight that; circulating insulin level may be a deciding factor for initiation of metformin therapy in AD. Metformin may be indicated in those with adequate amount of circulating insulin present, whereas, in insulin deficit population, it may lead to increase in Alzheimer's disease activity. This point needs further clarification from population based study.

Mechanism of therapeutic action of metformin:

In our present study, for evaluation of pathways involved in molecular mechanism of metformin in AD, molecular parameters e.g. brain level of p-AKTser473, p-ERK, GSK-3β was evaluated.

In present study disease control group, the expression of p-AKTser473 decreased as compared to sham. Treatment with positive control, metformin (50mg/kg, 100mg/kg, 150mg/kg) including SLN-Met50mg/kg, increased p-AKTser473 expression. No significant difference was observed among different treatment arms. Although no dose dependent effect was seen, but all the treatment arms showed increased the expression of p-AKTser473 as compared to disease control. Our findings are supported by findings of Kong LN et al., who demonstrated that, the ICV injection of Aβ (ICV- Aβ) leads to decrease in level of apoptosis associated protein AKT. AKT can increase the cell survival by reducing the Aβ toxicity.(57, 58)Activated AKT phosphorylate proapoptotic protein Bad, stimulate anti-apoptotic Bcl-2 expression and enhance cell survival. (57, 59) AKT also phosphorylates cAMP-response element-binding (CREB) protein, which enhance the transcription of anti-apoptotic genes Bcl-2.(60)

Study by Cruz E et al., claimed that increase intra-neuronal (Aβ1–42) accumulation causes the significant reduction in phosphorylation of ERK 1/2.(61) In our study, the disease control group showed higher brain level of Aβ1–42 and lower level of p-ERK as compared to sham. Treatment with metformin (150mg/kg) for 21 days significantly increased the level of p-ERK as compared to disease control group. Hippocampal ERK plays a vital role in neuronal-plasticity and memory.(62, 63) In insulin signaling downstream cascade, tyrosine kinase (surface receptor) activated by binding of IRS1 or IRS2 to initiate multiple pathway including ERK.(64) In the hippocampus, the ERK pathway is important for long term potentiation (LTP) and consolidation of memory as this is linked with a number of other signaling cascades like CaMKII, PKA, and PKC.(62, 65, 66)ERK activation occurs through multiple receptors and

second messengers which include insulin receptor or tyrosine kinase receptor through the PI3K pathway. (22)Insulin stimulation increases ERK activation.(67)IRS-1 play a vital role in signal transmission from upstream receptors to intracellular PI3K/AKT and ERK pathways.(68)

GSK3β is implicated in tau hyperphosphorylation and subsequently induce AD pathogenesis. In present study Aβ1–42 disease control groups showed significantly higher brain level of GSK3β. Treatment for 21 days with positive control (memantine), metformin (50mg/kg, 100mg/kg and 150mg/kg) and SLN-Met50mg/kg significantly decreased the level of GSK3β in model. Among all the treatment arm i.e. positive control (memantine), metformin (50mg/kg, 100mg/kg and 150mg/kg) and SLN-Met50mg/kg, there was no significant difference in GSK3β level. So, to summarize, treatment with metformin was associated with a decrease GSK3β level but the action was not dose dependent. Findings of our study are supported by Sarfstein R et al., they demonstrated that metformin decreases GSK3β levels in USPC-2 cells.(69)

In immunohistochemistry studies, in present study, the disease control group showed increase in Bax expression in brain. Treatment with positive control (memantine), metformin (50mg/kg, 100mg/kg and 150mg/kg) and SLN-Met50mg/kg decreased the expression of Bax. Bax is a marker for apoptosis. So, metformin causes decreased in expression of Bax, which can have a role in neuroprotection in AD.

Coming to the expression of Bcl2 expression by IHC, in the present study, we found that, the disease control decreases the expression of Bcl-2 as compared to Sham. But the treatment with positive control (memantine), metformin (50 mg/kg, 100mg/kg and 150mg/kg) and SLN-Met50mg/kg increased the Bcl-2 expression. Study by Chen D et al., also supports our finding.(70)

In H& E studies, in present study, disease control showed increase in neuronal injury score (as evident by higher number of pyknotic neurons) in the rat brain as compared to sham. Although, treatment with positive control (memantine), metformin (50mg/kg, 100mg/kg and 150mg/kg) and SLN-Met50mg/kg decreases neuronal injury score as compare to the disease control, but the difference was not statistically significant. No statistically significant difference was observed when different treatment groups were compared to sham and positive control as indicated in the representative microphotograph Unsal et al., demonstrated that ICV-STZ increases the number of apoptotic neurons in brain.(71)Mitochondrial permeability increase the cellular death and treatment with metformin inhibit the permeability of mitochondria and prevent the cell death.(72) Wang J et al., suggested that metformin enhance the adult neurogenesis *in vivo* and increase the spatial memory formation.(19)

To summarize the molecular mechanism of therapeutic efficacy of metformin, in our study, metformin treatment was associated with increase in level of phosphorylated ERK, phosphorylated AKT, decreased GSK-3β level, decreased Aβ and decreased hyperphosphorylated tau level. In Immunohistochemistry, metformin treatment was associated with increase in Bcl-2 expression and decrease in Bax expression and in histopathology, decrease neuronal injury score was seen. These findings highlight the involvement of the AKT/ERK/GSK-3β pathway in the therapeutic action of metformin in AD. These findings highlight the neuroprotective action of metformin. Regarding neuroprotective effect of metformin, in our study,

hippocampal and cortex neuronal protection was observed in metformin treated animals as evidenced by decreased pyknotic neurons in hippocampus, decreased neuronal injury score, increase in Bcl2 and decrease in Bax expression. Graphically the mechanism of protection of metformin is showed in **Figure-12.**

Conclusion:

On concluding the findings of our study, single ICV administration of Aβ, increased memory impairment, Aβ (1-42), hyperphosphorylated tau and neurodegeneration (apoptosis and neuronal injury), and these findings validates model as model of AD. In our study we observed that the ICV-Aβ lead to down-regulation of the p-AKTser473, p-ERK and up-regulation of the GSK-3β level.

21 days treatment in AD with metformin conventional and nanoformulation improved the memory parameter and reduced the level of Aβ (1-42), hyperphosphorylated tau and neurodegeneration (in histopathology) which are the culprit factors for AD pathogenesis.

Metformin (conventional and nanoformulation) treatment increased the p-AKTser473, p-ERK level and decreases the GSK-3β level, which establish its role via AKT, ERK, GSK-3β pathway, which highlights the involvement of this pathway in molecular mechanism of efficacy of metformin in AD.

Abbreviations

AD: Alzheimer's disease, AB: Amyloid beta, DM: diabetes mellitus, GSK: Glycogen synthase kinase, SLN: Solid lipid nanoformulation, Met: Metformin.

Declarations

Ethical Approval: The study protocol was approved by Institutional Animal Ethics Committee (Ref. No. 86/IAEC/581).

Consent to participate: Not applicable

Consent for publication: All authors read and approved the final manuscript.

Availability of supporting data: All the data provided in manuscript**.** The first author and the corresponding authors have full accessibility to study data. In case of any clarification, the corresponding author may be contacted.

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Authors' Contributions:

All authors read and approved the final manuscript.

HK: Hypothesis generation, Protocol making, Ethical approval, conduct of experiment, statistical analysis, interpretation of results, manuscript writing, manuscript approval.

AC: Overall guidance,Hypothesis generation, Protocol making, Ethical approval, interpretation of results, manuscript approval.

PS: Interpretation of results, statistical analysis, manuscript writing, manuscript approval.

MM: Guidance,Hypothesis generation, Protocol making, interpretation of results, manuscript approval

DB: Overall guidance,Hypothesis generation, Protocol making, interpretation of results, manuscript approval

BDR: Overall guidance,Hypothesis generation, Protocol making, Histopathology,Ethical approval, interpretation of results, manuscript approval.

AB: Histopathology,interpretation of results, manuscript approval.

BM: Overall guidance,Hypothesis generation, Protocol making, Ethical approval, interpretation of results, manuscript approval.

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Figures

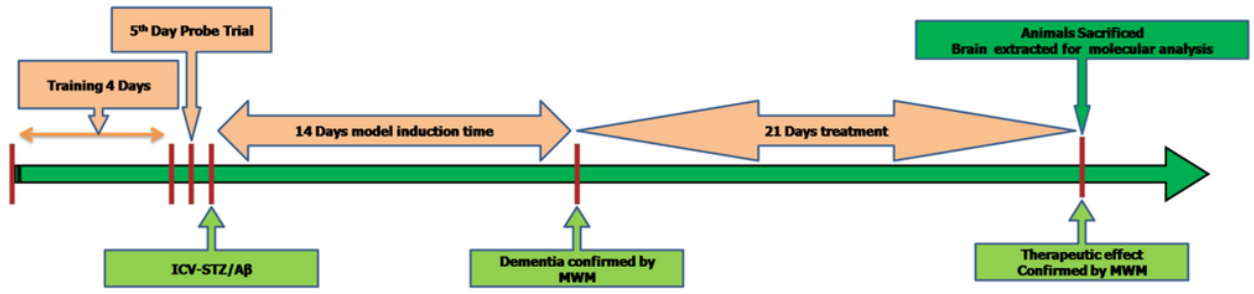


Figure 1

Details of experimental timelines.

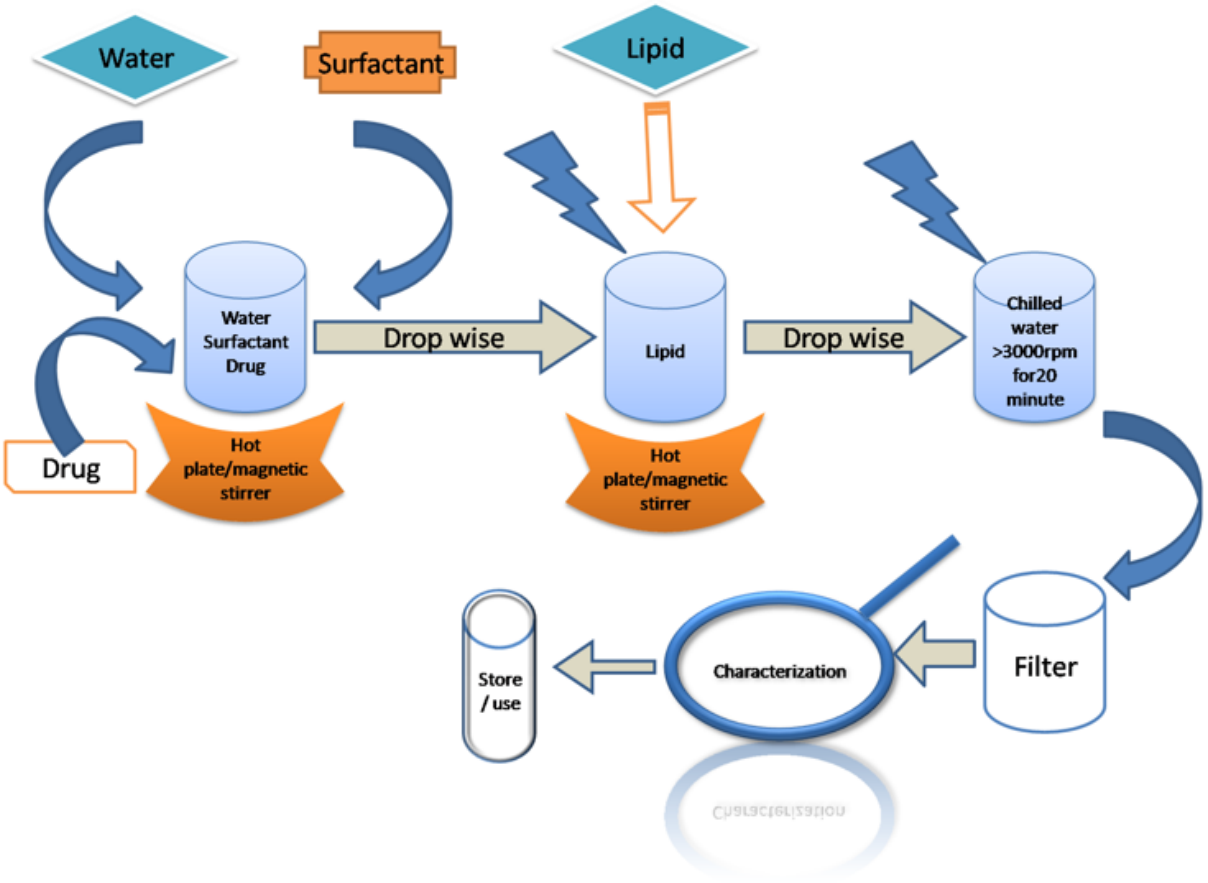


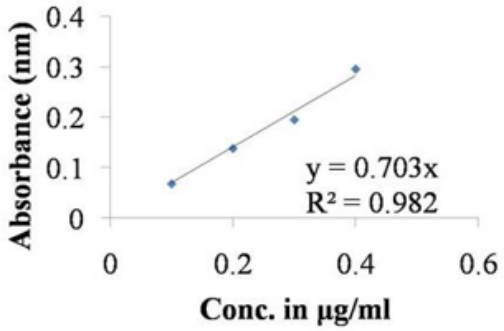
Figure 2

Showing the flow of events in preparation of the SLN nanoformulation of metformin. 400 mg of Pluronic F-127 was added to 5 ml of distilled water on the hot plate with magnetic stirrer. After dissolution of the surfactant, 500 mg metformin HCl was added into that solution and magnetic stirring was continued on hot plate. At the same time 100 mg compritol (lipid) was added in another beaker and melted on another magnetic stirrer with hot plate. After melting of compitrol, the drug surfactant mixture solution was added drop-wise with the help of pipette to the beaker containing melted compritol and vigorous stirring was maintained while adding the solution. Then this mixture was added drop-wise to the chilled (4oC) distilled water (remaining volume upto 25ml). Stirring was maintained with the mechanical stirrer continuously at more than 3500 rpm atleast for 20 minutes and that solution was filtered.

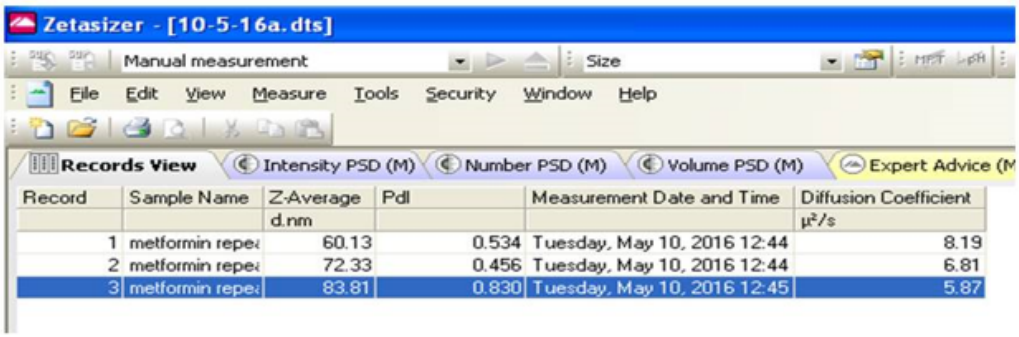
A.1

Conc. µg/ml	Absorbance (232nm)
0.1	0.068
0.2	0.138
0.3	0.195
0.4	0.295667

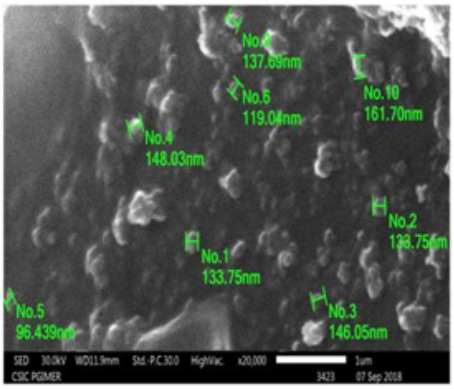
A.2



B.



C.1



C.2

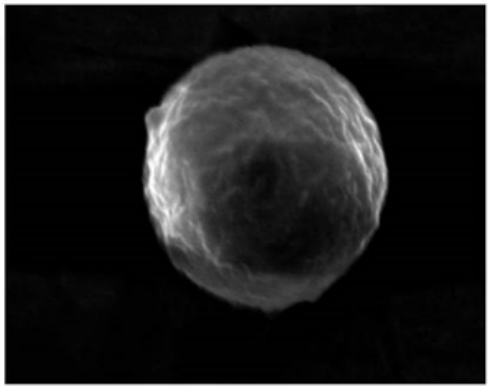


Figure 3

Details of solid lipid nanoformulation of metformin. A.1.: Metformin standard curve (UV spectrophotometer) A.2. Showing standardization of nanoformulation loading by the spectrophotometer. Dilutions and respective absorbance (232nm) of metformin in spectrophotometer was used to plot standard curve. B. Showing particle size by zetasizer. C. Picture in Scanning Electron Microscopy (magnification x20000), showing spherical shape, and size (<200nm in diameter) of the SLN nano-particles.

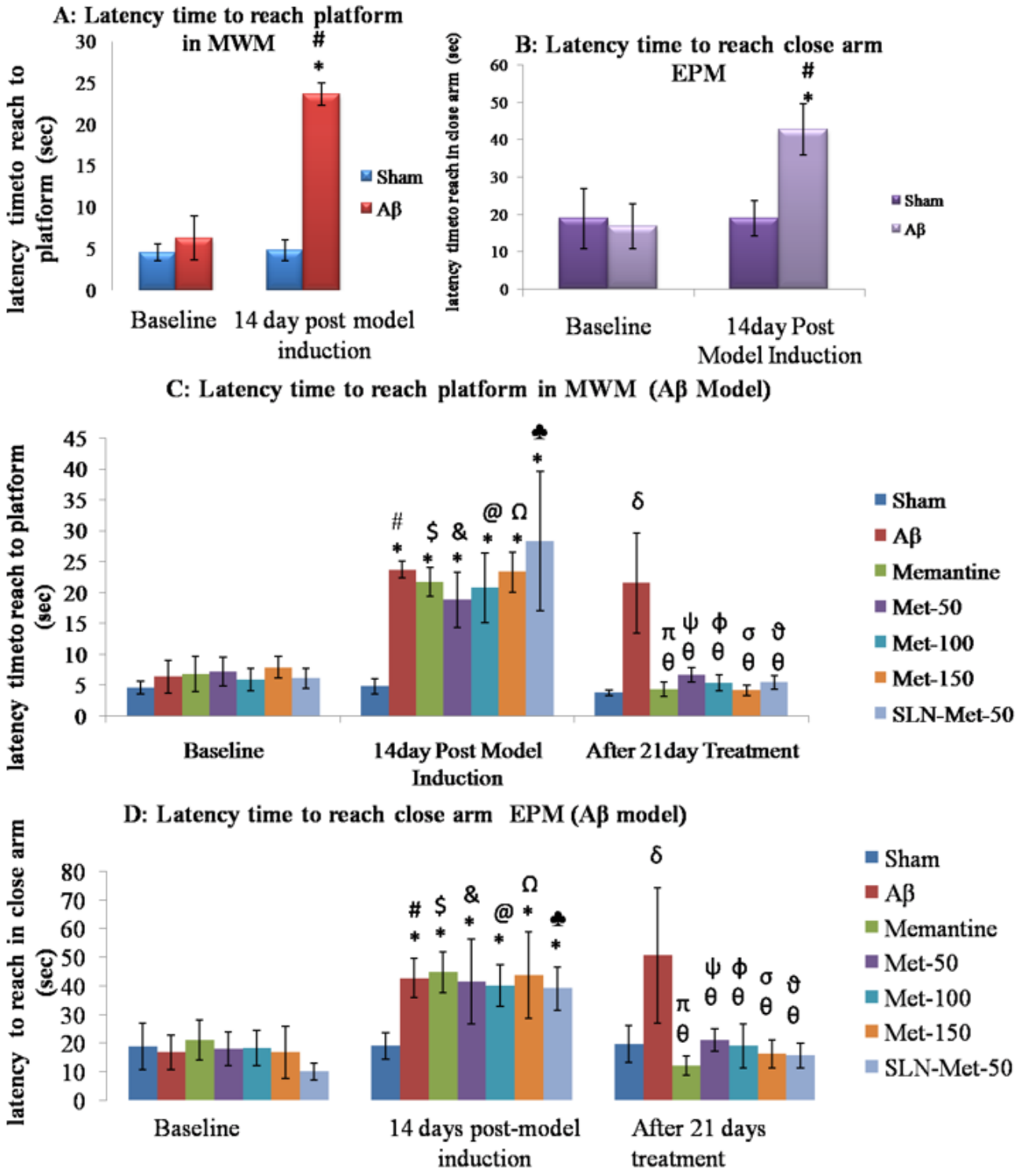


Figure 4

Figure A: latency time to reach platform in MWM, Figure B: latency to reach close arm in EPM in Aβ Model (data represented as mean ± SD). *signifies p<0.05 when compared to Aβ group (baseline). # signifies p<0.05 when compared to sham group at 14 day post model induction. Figure C: Showing latency time to reach platform in MWM in Aβ Model, in which data represented as (mean±SD). Figure D. Showing latency time to reach close arm EPM in Aβ model, in which data represented as (mean±SD). *p<0.05 compared to

Sham after 14 days Post-Model induction, #p<0.05 when compared to (Aβ assigned group) premodel induction (baseline), $p<0.05 when compared to (Memantine assigned group) premodel induction (baseline), & p<0.05 when compared to (Met50 assigned group) premodel induction (baseline), @p<0.05 when compared to (Met100 assigned group) premodel induction (baseline), Ω p<0.05 when compared to (Met150 assigned group) premodel induction (baseline),♣ p<0.05 when compared to (SLN-Met-50 assigned group) premodel induction(baseline), δ p<0.05 compared to Sham after 21 days treatment period (Post-Model Treatment), θ p<0.05 when compared to (Aβ group) 21 days treatment period (Post-Model Treatment), π p<0.05 when compared to (Aβ -Memantine assigned group) after14 days (Post-Model induction), ψ p<0.05 when compared to (Aβ -Met50 assigned group) after14 days (Post-Model induction), φ p<0.05 when compared to (Aβ -Met100 assigned group) after14 days (Post-Model induction), σ p<0.05 when compared to (Aβ -Met150 assigned group) after14 days (Post-Model induction),ϑ p<0.05 when compared to (Aβ-SLN-Met-50 assigned group) after14 days (Post-Model induction)

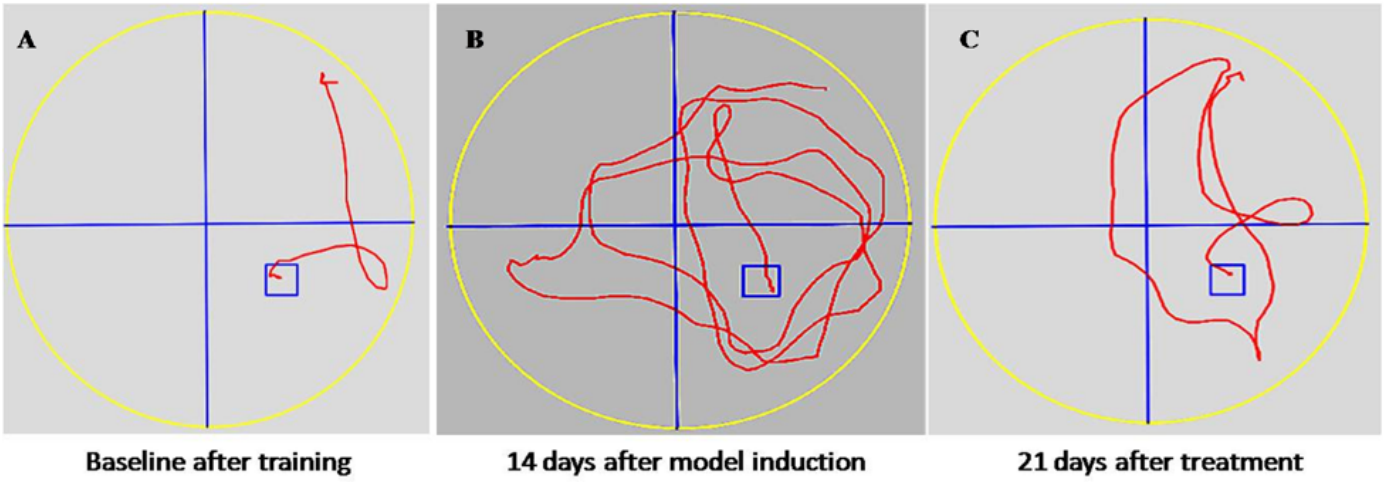


Figure 5

Showing the path pattern of animals to reach to the platform in MWM; (A) Baseline after training, (B) After model induction, (C) After treatment.

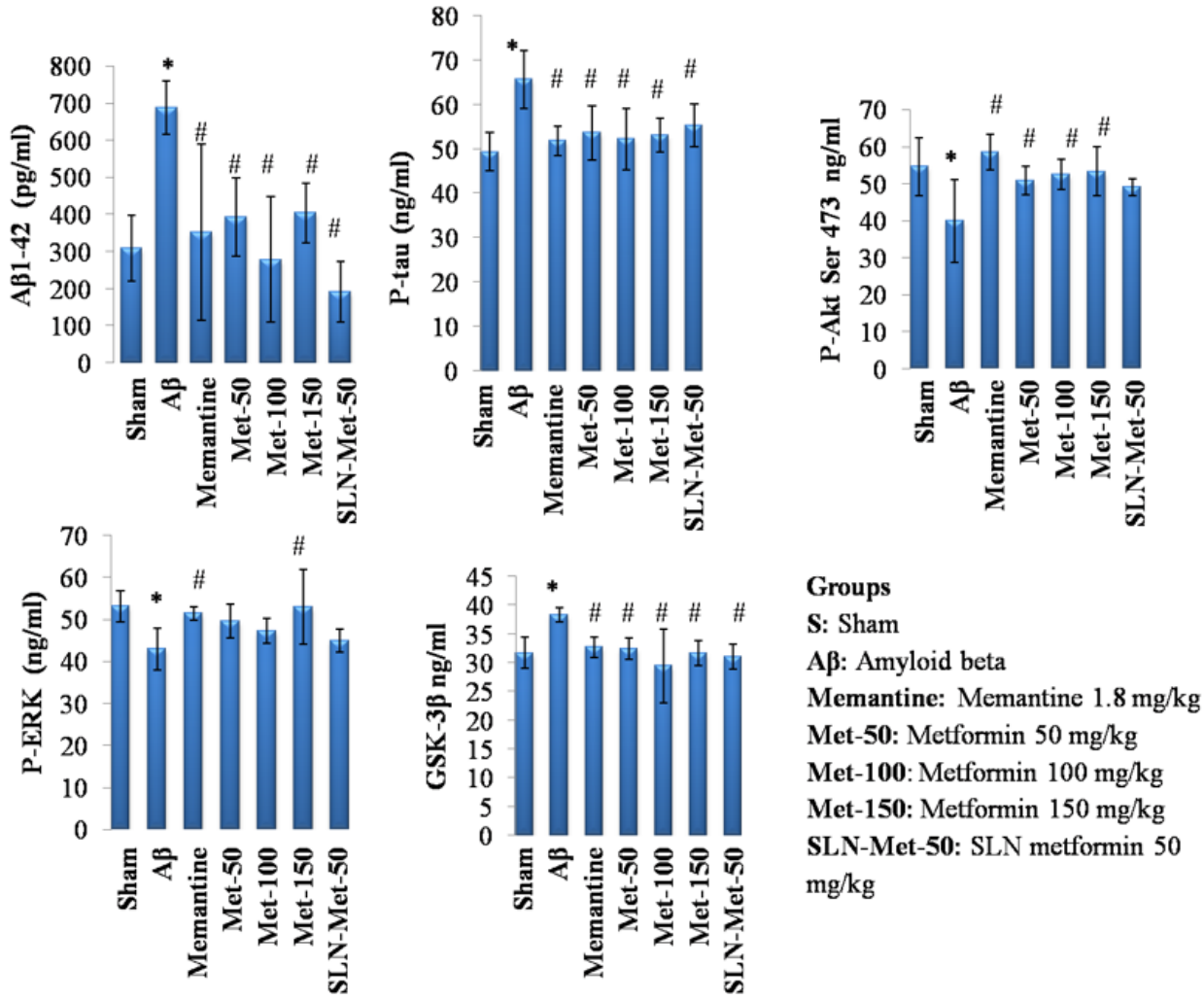


Figure 6

Molecular parameters in brain homogenate. A: Aβ1-42 level in brain homogenate B: P tau level C: P-Akt Ser 473level, D: P-ERK level, E: GSK-3B. Data represented as (mean ± SD). *p<0.05 compared to sham, * p<0.05 compared to sham, #p<0.05 when compared to Aβ group, $p<0.05 when compared to Memantine group, &p<0.05 when compared to Met50 mg/kg group, @p<0.05 mg/kg when compared to met 100 mg/kg, Ω p<0.05 when compared to Met150 group, ♣ p<0.05 when compared to Aβ-SLN-Met50mg/kg group.

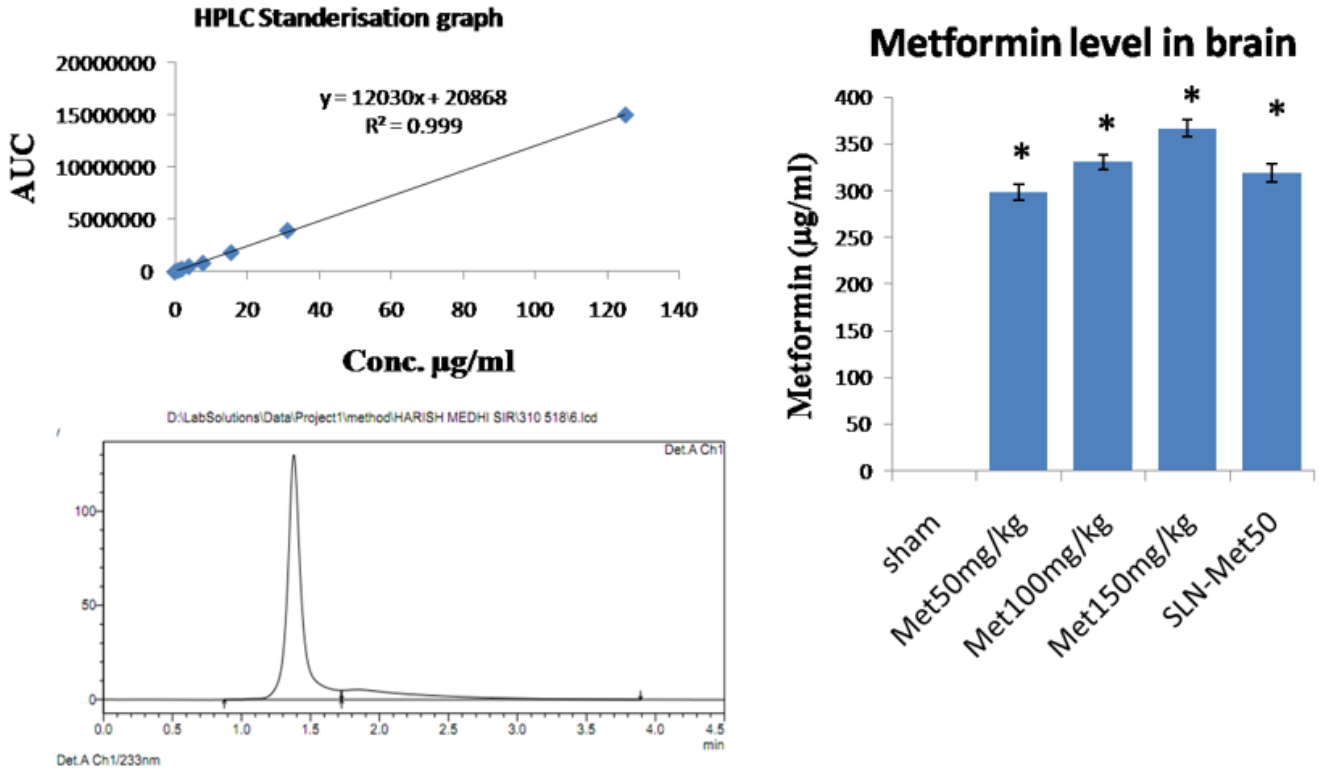


Figure 7

Determination of metformin by HPLC.

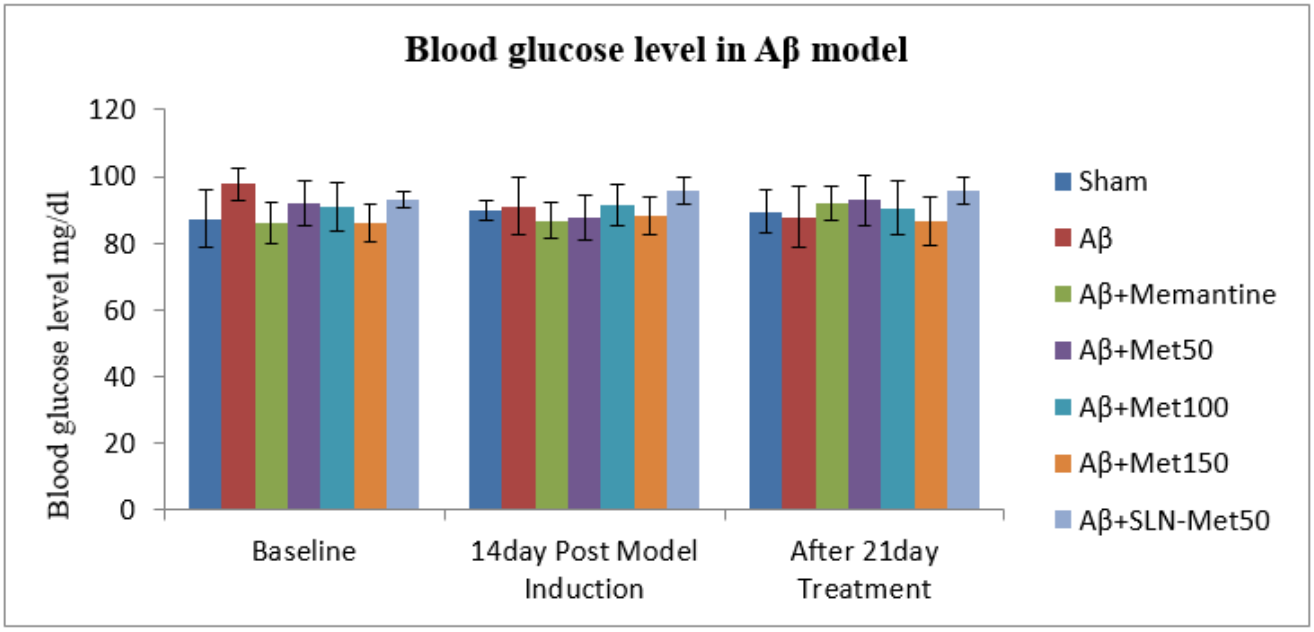


Figure 8

Showing blood glucose level in Aβ model, in which data represented as (mean ± SD), *p<0.05 compared to sham, #p<0.05 when compared to Aβ group, $p<0.05 when compared to Memantine group, & p<0.05 when compared to Met50 group, @p<0.05 when compared to met 100, Ω p<0.05 when compared to Met 150 group, ♣ p<0.05 when compared to Aβ-SLN-Met50 group.

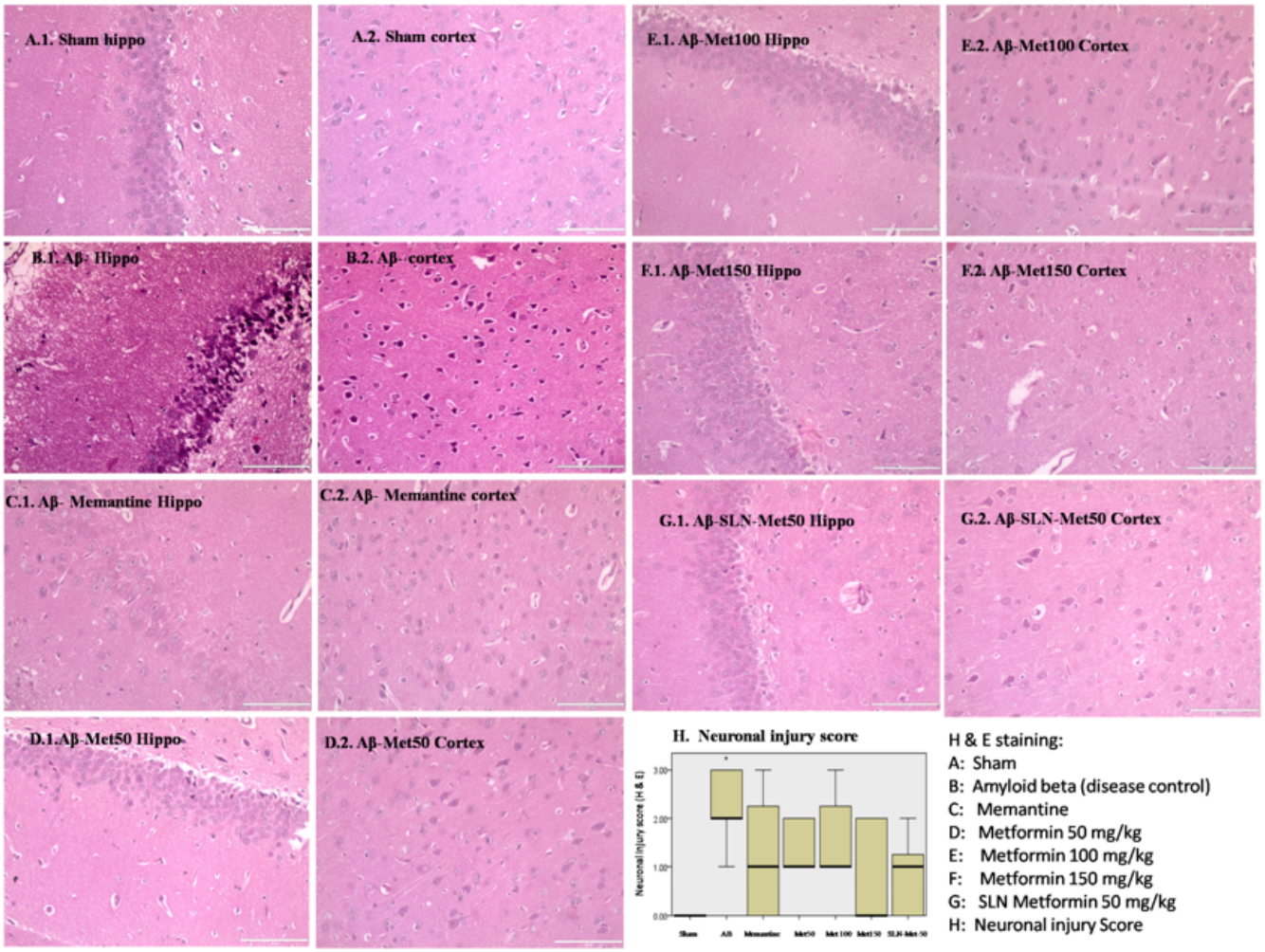


Figure 9

Representative microphotograph showing Hematoxylin–Eosin stained brain sections of Aβ model (hippocampus and cortex: magnification 40X); Box and whisker plot showing neuronal injury score Aβ model, in which data represented as (Median, IQR, Range),*p<0.05 compared to sham, #p<0.05 when compared to Aβ group, $p<0.05 when compared to Memantine group, & p<0.05 when compared to Met50 group, @p<0.05 when compared to met 100, Ω p<0.05 when compared to Met150 group, ♣ p<0.05 when compared to Aβ-SLN-Met-50 group.

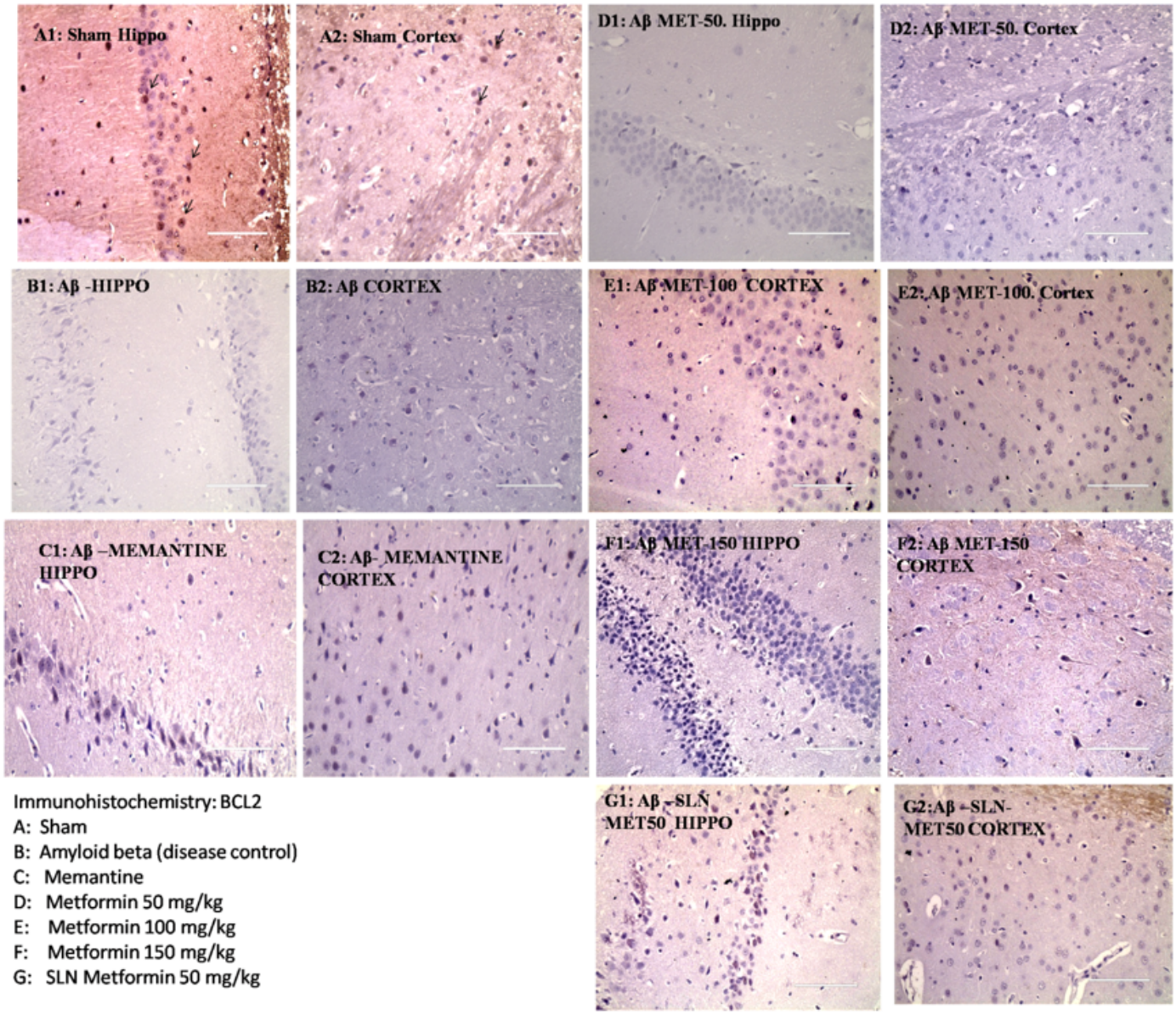


Figure 10

Representative microphotograph pictures of brain section (hippocampus and cortex) of Aβ model (magnification 40 X);

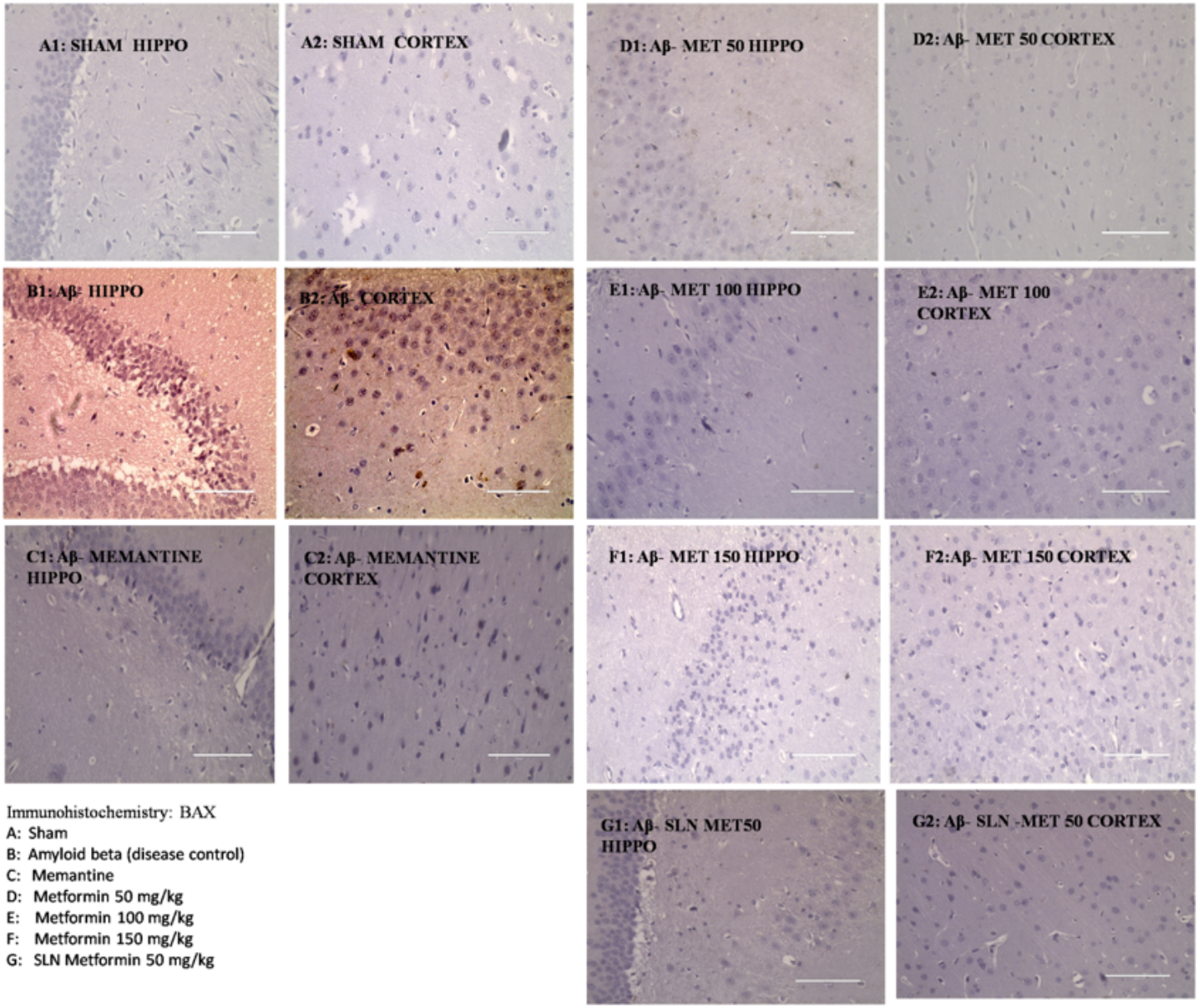


Figure 11

Representative microphotograph of brain sections of Aβmodel (hippocampus and cortex, magnification 40X).

Figure 12

Showing effect of metformin in AD