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Original article

Alpha lipoic acid and metformin alleviates experimentally induced insulin resistance and cognitive deficit by modulation of TLR2 signalling

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ABSTRACT

Background: Obesity is commonly found to be co-morbid with type 2 Diabetes Mellitus. In obese diabetic patients, TLR-2 receptor induced inflammation leads to the development of insulin resistance (IR). Furthermore, the IR is considered to be the most important cause for promoting cognitive decline which is evident in brain of patients with Alzheimer's disease related dementia (ADRD).
Methods: In this study, the effect of α-lipoic acid (ALA) has been examined in rodent model of zymosan induced insulin resistance and cognitive deficits, targeting at TLR-2 signalling. TLR-2 agonist, Zymosan initiates inflammatory cascade, resulting in IR and cognitive dysfunction. Zymosan (50 mg/kg *ip*) was given to mice on 1st, 8th, 15th and 22nd day to induce IR which was confirmed by hyperglycaemia, hyperinsulinemia, hyperlipidimea, increased glycated haemoglobin and HOMA-IR. Further the cognitive performance was assessed in Morris water maze revealing cognitive deficit in zymosan treated mice.
Results: Daily treatment with ALA for 28 days (50, 100, 200 mg/kg, *ip*) significantly improved insulin sensitivity and cognitive performance in mice by decreasing insulin resistance, corticosterone, IL-6 levels, acetylcholinesterase enzyme activity and oxidative stress in liver, cortex and hippocampus. ALA also increased adiponectin level and reduced body weight. Combination of ALA (100 mg/kg, *ip*) with metformin (100 mg/kg, *ip*) exhibited a potentiating effect in improving cognitive performance and insulin signalling.
Conclusion: The findings of the study supported the hypothesis that TLR-2 induced inflammation leads to insulin resistance and cognitive impairment and provides an evidence for the therapeutic effect of ALA in IR and ADRD patients.

Introduction

Type 2 diabetes mellitus (T2DM) and obesity are the diseases of major concern at present. Both of these pathological conditions are found to be co-morbid, with a high prevalence worldwide. T2DM is associated with various complications that can degrade the quality of life of a patient. Alzheimer's disease related dementia (ADRD) is a pathological condition that is found to be significantly co-morbid with T2DM [1]. Moreover the connection between obesity-T2DM-cognition has been studied widely by researchers.

Human insulin is a 51amino acid containing peptide hormone, synthesised by β-cells of pancreas which acts through the IRS–Akt and MAPK signalling pathways. It maintains the plasma glucose level by promoting glucose uptake by cells and suppressing glucose production and release in liver [2]. The reduced sensitivity to insulin action in body tissues is known as insulin resistance (IR). Insulin receptors are also expressed in the brain, with variable expression in different regions [3].

On the other hand Toll-like receptors (TLRs) are highly specialised recognition receptors which navigate the innate inflammatory response to various foreign substances. TLR2 identifies various derived products of microorganisms such as, lipoproteins, peptidoglycan and lipoteichoic acid etc. [4]. Significant increase in TLR2 is also observed at both mRNA and proteins levels in subjects with T2DM [5]. Activation of TLR2 and JNK mediated signalling pathways promote inflammation and cellular

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insulin resistance. The insulin sensitivity is increased by the inhibition of TLR2 expression in mice that are diet induced obese [6]. Also, the TLR2 knockout mice showed protective effect against IR. When adipocytes obtained from obese people were examined, the upregulated expression of IL-6 was observed and it was further correlated with the IR [7]. Hence elevated TLR2 expression in obese patients is directly linked to inflammation [8] and chronic inflammation in obesity is an important cause to induce IR. These reports suggest a possible link between IL-6 levels and obesity in the development of T2DM and also suggest that IL-6 is an important biomarker for detecting early risk of T2DM. TLRs also contribute to initiation of inflammatory response in microglia and thus promotes AD pathogenesis [9]. Hence TLR2 induced inflammatory cascade could be a possible mechanism for the development of insulin resistance and T2DM in obese patients. Insulin resistance is the core feature of T2DM and research has shown that it also occurs in cognitive deficit patients with ADRD [10]. Further, obesity and high fat diet induced metabolic syndrome has been found to be linked with the release of proinflammatory cytokines. These inflammatory markers can cross the BBB and have been reported to cause brain insulin resistance. So, obese people are highly prone to suffer from cognition and memory impairment [11]. The above mentioned facts show strong correlation between obesity-T2DM-inflammation-Alzheimer's disease.

Obesity leads to activation of TLR2 induced cascade, leading to abnormal insulin signalling, so the animal model we used in this study was zymosan induced insulin resistance. Zymosan is a mixture of cell wall particles from the *Saccharomyces cerevisiae* yeast. The zymosan is a TLR2 agonist and it binds to the extracellular TLR2 domain to initiate a strong inflammatory response [12] that led to peripheral IR and cognitive deficits in the present study. The development of IR and inflammation by zymosan has been confirmed in various research studies [13–15].

α-Lipoic acid (ALA), a natural compound, is chemically 1,2-dithiolane pentanoic acid. It is an analogue of octanoic acid and synthesised in mitochondria. ALA has potent anti-inflammatory [16] and antioxidant properties [17]. Decreased ALA level has been found in patients with T2DM. Various studies have shown that the ALA can efficiently improve insulin sensitivity and reverse the IR [18–20]. Moreover, ALA have been reported to decrease the gene expression of TLR2 [21]. The same TLR2 is responsible for insulin resistance [22], which in turn leads to loss in memory and cognitive deficit [23]. So, considering all these evidences together we made this hypothesis that ALA could be used as an adjunct for insulin resistance and ADRD therapy as it can attenuate development of IR by suppressing inflammation and oxidative stress. Thus, the objective is to modulate TLR-2 induced alteration in insulin signalling using ALA to improve insulin sensitivity and cognitive function.

Materials and methods

Animals

Albino LACA mice (male) weighing around 20–30 g were obtained from the institute Animal House. Standard housing conditions for the mice were maintained with temperature (25±2 °C), relative humidity (40±10%) and 12 h period of dark and light alternatively. Normal animal feed and tap water were given to the mice regularly. All the experiments related to animal handling, care and maintenance in this protocol were approved by the Institutional Animal Ethics Committee (IAEC) (Regd. No.45/GO/ReBI/S/99/CPCSEA) and performed in accordance to the guidelines of National Science Academy, for the experimental as well as care of animals.

Experimental protocol

All the animals were randomly divided into following seven groups: naïve (normal saline); control (zymosan 50 mg/kg); ALA 50 (zymosan 50 mg/kg + alpha lipoic acid 50 mg/kg); ALA 100 (zymosan 50 mg/kg + alpha lipoic acid 100 mg/kg); ALA 200 (zymosan 50 mg/kg + alpha lipoic acid 200 mg/kg); Met 100 (zymosan 50 mg/kg + metformin 100 mg/kg); ALA 100 + Met 100 (zymosan 50 mg/kg + alpha lipoic acid 100 mg/kg + metformin 100 mg/kg). Mice received 50 mg/kg zymosan intraperitoneally (*ip*) once per week for 28 days i.e. on day 1, 8, 15, and 22 to induce insulin resistance. Zymosan was given as solution in 0.9% w/v saline. Initially, zymosan 100 mg/kg was used based on the previous study [24]. Since 100 mg/kg produced 90% mortality so the dose was reduced to 50 mg/kg for further studies [14] ALA was administered *ip* daily for 28 days, starting from day 1 as suspension in 0.1% w/v carboxymethyl cellulose. Metformin was given *ip* as solution in distilled water daily for 28 days, starting from day 1 (Fig. 1). Alpha lipoic acid and metformin doses were selected based on previously reported studies [25,26].

Measurement of animal body weight

Body weights of animals were recorded every week throughout the experimental period.

Biochemical estimations

Blood was isolated from retro-orbital plexus of mice. Blood was kept for some time to clot and then centrifugation was done at 2000 *g* for 10 min. The watery pale yellow coloured serum was separated and stored at a freezing temperature of -20 °C till further estimations.

Measurement of glucose and glycosylated haemoglobin levels

Serum glucose levels of 6 h fasted mice were measured on day 0, 7, 14, 21 and 28 by glucose oxidase peroxidase (GOD-POD) method using a diagnostic kit (Erba Glucose Kit, Transasia Bio-Medicals Ltd., Solan, India). Glycosylated haemoglobin (HbA1c) levels of 6 h fasted mice were measured in the blood sample on day 28 using a diagnostic kit (Excel Diagnostics, Hyderabad).

Measurement of serum insulin levels and insulin resistance index

On 28th day, levels of insulin were estimated by ELISA Kit (Crystal Chem Inc). A physiological parameter, HOMA (Homeostasis Model Assessment)-IR index, a marker of insulin resistance was determined applying the following formula [27].

HOMA-IR = fasting insulin (micro U/ml) × fasting glucose (mg/dl) / 405

Measurement of serum lipid profile

Serum cholesterol levels were measured on day 28 using CHOD-PAP method using diagnostic kit (Erba cholesterol Kit, Transasia Bio Medicals Ld., Solan, India). Triglycerides levels were measured in serum using GPO-trinder method (Erba triglycerides kit Transasia Bio Medicals Ltd., Solan, India).

Measurement of IL-6 and adiponectin levels

The quantification of serum IL-6 was done on day 28 as per instructions of RayBio® Mouse IL-6 Assay ELISA kit. The ELISA kit

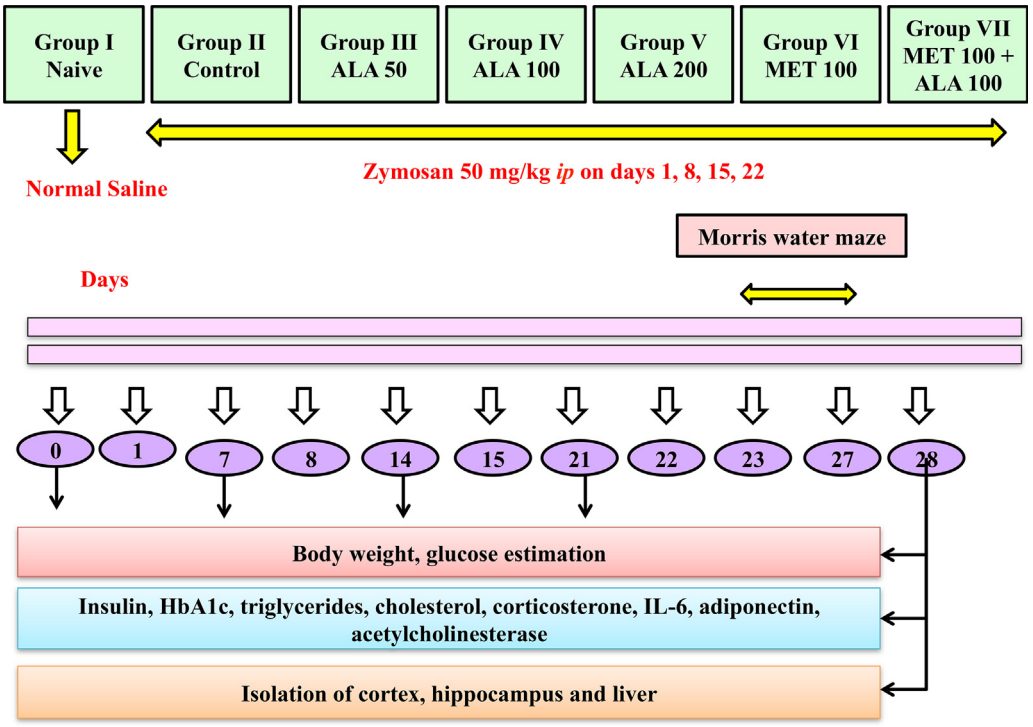

Fig. 1. Experimental protocol.

(RnD Systems) employing the quantitative sandwich enzyme immunoassay technique was used for measuring serum adiponectin levels.

Measurement of serum corticosterone levels

Assay for corticosterone was carried out on day 28 according to the modification of Silber's method [28] described by Rinwa and Kumar [29].

Morris water maze test for assessment of learning, memory and cognition

Morris water maze was performed on day 23–27 to find out the effect of zymosan, a TLR2 agonist on cognitive behaviour of the animals. The maze used was a circular tank (105 cm in diameter and 30 cm in height) filled upto 15 cm with water. There was a square shaped platform (10 cm in diameter, 15 cm in height) at the centre of the 4th quadrant, 1–2 cm below the water surface. The maze was kept in a spacious room, having some visual cues for animals. Each animal was given four trials each day for four successive days. In trial days, each mouse was put in one of the quadrant of the water tank with face towards the wall, and the time was noted down for the mouse to locate the hidden platform referred as escape latency of the animal. The mice were placed on the platform for 10–15 s and then taken back to its home cage till the next trial. If the mice could not locate the platform in 90 s time, then it was manually directed to the platform with a stick. Latency to locate the hidden platform was noted for each of the trials. Probe test was carried out on day 5 after removing the platform. Each mouse was allowed to swim for 90 s. The frequency of entries and time spent in the 4^{th} quadrant referred as target quadrant (quadrant of the hidden platform of the trial period), was calculated. All the performances were video recorded with a computer aided video tracking system (Ethovision 3.1 software) [30].

Measurement of oxidative stress parameters

Preparation of tissue homogenates

For estimation of biochemical parameters, all the animals were sacrificed on day 28. Liver, cortex and hippocampus were dissected and the homogenates of 10% (w/v) were prepared in phosphate buffer (0.01 M, pH 7.4). Centrifugation of the homogenates was done at 4000 *g* for 20 min at 4 °C and supernatants were separated for further estimation of reduced glutathione, catalase, lipid peroxidation, nitrite levels and acetylcholinesterase activity.

Measurement of protein

The protein concentrations were measured by using biuret method. The standard used was Bovine serum albumin (BSA) [31].

Measurement of lipid peroxidation

Lipid peroxidation leads to generation of its marker malondialdehyde, which was estimated in the homogenates of liver, hippocampus and cortex. For this, tissue homogenate (0.5 ml) and tris–hydrochloride (0.5 ml) were mixed and then kept for 2 h incubation at 37 °C. Then, 1 ml of trichloroacetic acid (10% w/v) was added to the mixture followed by cold centrifugation at 1000 *g* for 10 min. Supernatant was separated, 1 ml of thiobarbituric acid (0.67% w/v) was added to it and kept for boiling for 10 min. 1 ml of distilled water was added after cooling and absorbance was recorded at 532 nm using UV–vis spectrophotometer (Parkin Elmer, USA) [32]. Calculations were done using the molar extinction coefficient of chromophore as $1.56 \times 105\ M^{-1}\ cm^{-1}$ and expressed as nano moles per mg protein of malondialdehyde.

Measurement of reduced glutathione

Reduced glutathione (GSH) was measured as per the Ellman's method [33]. Precipitation of 1 ml of tissue homogenate was done with 1 ml of sulfosalicylic acid (4% w/v). The precipitated mixture was then kept at 4 °C for 1 h. Cold centrifugation was done at 1200 *g* for a period of 15 min. 1 ml supernatant was separated,

2.7 ml of 0.1 M phosphate buffer (pH 8) was added to it followed by addition of 0.2 ml of 5,5-dithiobis (2-nitrobenzoic acid). Yellow colour was produced which was measured immediately at 412 nm using UV–vis spectrophotometer (Perkin Elmer, USA). Values were calculated using molar extinction coefficient of the chromophore as $1.36 \times 104\ M^{-1}\ cm^{-1}$ and units were shown as n moles of GSH per mg protein.

Measurement of catalase

Catalase assay was done as follows. A total 3 ml mixture containing 1.95 ml of 0.05 M phosphate buffer (pH 7.0), 1 ml of 0.019 M hydrogen peroxide (H_2O_2) and 0.05 ml of tissue homogenate was taken. Absorbance was recorded at 240 nm using a UV–vis spectrophotometer (Perkin Elmer, USA). Values of the results were expressed as μM of H_2O_2 decomposed per mg protein per min [34].

Estimation of nitrite

The nitric oxide spontaneously gets oxidized to nitrite and nitrate. For its assessment, tissue homogenate and Griess reagent were mixed in equal volumes. Griess reagent consists of equal quantity of 0.1% naphthyl ethylenediamine dihydrochloride and 1% sulphanilamide in 5% phosphoric acid. This mixture was then incubated at room temperature in a dark place for 10 min. Absorbance of the mixture was read at 540 nm with a UV–vis spectrophotometer (Perkin Elmer, USA). Concentration of the nitrite was found out using sodium nitrite as standard from a standard curve. Values were reported as a μM per ml [35].

Estimation of acetylcholinesterase activity

Acetylcholinesterase, an enzyme catalyzing acetylcholine breakdown is a biomarker for cholinergic neuron function in brain. Its assessment was done in the hippocampus and cortex as per the Ellman's method [36]. The assay mixture consisted of 50 μl of tissue homogenate, 3 ml of sodium phosphate buffer (pH 8), 100 μl of acetylthiocholine iodide (ATChI), and 100 μl of DTNB (Ellman's reagent). The change in absorbance was recorded at 412 nm for 2 min period at an interval of 30 s with a UV–vis spectrophotometer (Perkin Elmer, USA). Calculations were done and values were represented as μM of acetylthiocholine iodide hydrolyzed per mg protein per min.

Statistical analysis

Statistical analysis was done by One Way ANOVA followed by Tukey's test, Two Way ANOVA followed by Bonferroni's *post hoc* test with the help of graph pad prism. All the data values were shown as mean ± SEM, and statistical significance was taken at $p < 0.05$.

Results

Effect of ALA treatment and its combination with metformin on body weight

Zymosan (50 mg/kg) administration in mice once a week for 28 days produced significant increase in the body weight as compared to naïve group (Table 1). Treatment with different doses of ALA and metformin significantly attenuated the increase in body weight on day 21 and 28 as compared to zymosan treated group. Combination treatment with [Met (100) + ALA (100)] also significantly attenuated the gain in body weight from day 14 day to 28. However, the change in the body weight produced by all treatments was not significantly different.

Effect of ALA treatment and its combination with metformin on fasting blood glucose levels

Zymosan produced marked hyperglycaemia characterized by significant increase in serum fasting glucose levels as compared to naïve group on day 7, 14, 21 and 28. Administration of ALA (50 mg/kg, 100 mg/kg and 200 mg/kg) and metformin (100 mg/kg) significantly decreased the developed hyperglycaemia from day 7 onwards. The effect produced by ALA (200 mg/kg) is significant as compared to standard drug metformin in the last two weeks. In addition, combination treatment (Met (100) + ALA (100)) produced significant reduction in elevated glucose levels on day 7, 14, 21 and 28 as compared to zymosan group and their effects *per se* (Table 2).

Effect of ALA treatment and its combination with metformin on serum total cholesterol and triglycerides levels

Total cholesterol and triglycerides levels in zymosan injected group were markedly and significantly higher than the corresponding values in naïve animals on day 28. Treatment with ALA (100 mg/kg and 200 mg/kg) and metformin (100 mg/kg) significantly inhibited the zymosan induced rise in cholesterol and triglycerides levels whereas ALA (50 mg/kg) was found to be effective in significantly decreasing cholesterol levels only. ALA (200 mg/kg) was found to be more effective in decreasing cholesterol and triglycerides levels than metformin treatment. In addition, treatment with [Met (100) + ALA (100)] combination significantly decreased the rise in plasma cholesterol and triglycerides levels as compared to zymosan group and their *per se* effects (Fig. 2 and 3).

Effect of ALA treatment and its combination with metformin on insulin levels and HOMA-IR

Insulin levels and HOMA-IR index in zymosan injected group was significantly higher relative to naïve group. Treatment with

Table 1
Effect of ALA treatment and its combination with metformin on body weight.

Treatment (mg/kg)	Body weight (g)				
	Day 0	Day 7	Day 14	Day 21	Day 28
Naive	25.8 ± 0.66	26.0 ± 0.71	25.2 ± 0.97	25.6 ± 1.03	26.4 ± 0.51
Zymosan	26.0 ± 0.50	27.4 ± 0.37	29.0 ± 0.74[a]	31.8 ± 0.61[a]	34.0 ± 0.65[a]
ALA (50)	25.4 ± 0.60	26.2 ± 0.49	26.6 ± 0.80	27.0 ± 0.55[b]	27.8 ± 0.49 [b]
ALA (100)	26.8 ± 0.58	27.2 ± 0.86	26.4 ± 0.93	26.1 ± 0.75 [b]	26.0 ± 0.77 [b]
ALA (200)	26.0 ± 0.82	26.5 ± 1.04	26.4 ± 0.85	26.5 ± 0.65 [b]	26.3 ± 0.63 [b]
Met (100)	26.8 ± 1.02	28.2 ± 1.02	28.6 ± 1.25	27.4 ± 1.12 [b]	26.6 ± 0.81 [b]
Met (100) + ALA (100)	27.0 ± 1.00	26.0 ± 1.00	25.5 ± 0.50[b]	25.0 ± 1.00 [b]	25.0 ± 1.00 [b]

All the data were expressed as the mean ± SEM; [a]$p < 0.05$ as compared to naive, [b]$p < 0.05$ as compared to zymosan; Two-way ANOVA followed by Bonferroni's post test. ALA: Alpha lipoic acid; Met: Metformin.

Table 2
Effect of ALA treatment and its combination with metformin on fasting blood glucose levels.

Treatment (mg/kg)	Fasting blood glucose levels (mg/dl)				
	Day 0	Day 7	Day 14	Day 21	Day 28
Naïve	101.8 ± 0.56	100.4 ± 0.37	101.7 ± 0.40	101.0 ± 0.73	100.5 ± 0.39
Zymosan	102.2 ± 0.64	112.1 ± 0.69[a]	126.4 ± 0.34 [a]	146.2 ± 1.00 [a]	163.8 ± 0.70 [a]
ALA (50)	102.4 ± 0.25	110.4 ± 0.36	121.6 ± 0.53[b]	135.0 ± 0.50[b]	147.4 ± 0.63 [b]
ALA (100)	103.2 ± 0.41	108.0 ± 0.25 [b,c]	117.9 ± 0.49 [b,c]	124.1 ± 0.30 [b,c]	134.4 ± 1.18 [b]
ALA (200)	103.6 ± 0.46	106.3 ± 0.47 [b,c]	110.9 ± 0.46 [b,c,d]	119.7 ± 0.64 [b,c,d]	124.2 ± 0.84 [b,c,d]
Met (100)	102.9 ± 0.55	107.4 ± 0.25 [b]	111.8 ± 0.40 [b]	124.8 ± 0.42 [b,e]	132.1 ± 0.50 [b,e]
Met (100) + ALA (100)	104.0 ± 0.50	104.7 ± 0.75 [b,d,f]	109.9 ± 0.90 [b,d,f]	114.8 ± 0.80[b,d,f]	118.9 ± 0.90[b,d,f]

All the data were expressed as the mean ± SEM; [a]$p < 0.05$ as compared to naive, [b]$p < 0.05$ as compared to zymosan; [c]$p < 0.05$ as compared to ALA (50 mg/kg); [d]$p < 0.05$ as compared to ALA (100 mg/kg); [e]$p < 0.05$ as compared to ALA (200 mg/kg); [f]$p < 0.05$ as compared to Met (100 mg/kg); Two-way ANOVA followed by Bonferroni's post test. ALA: Alpha lipoic acid; Met: Metformin.

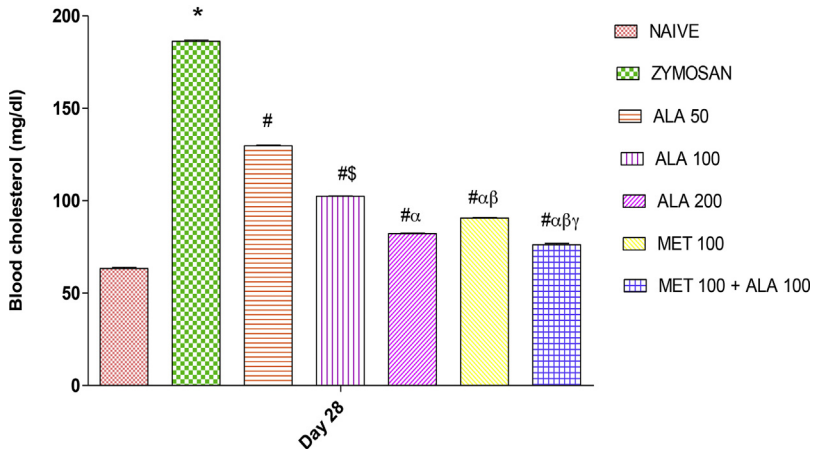

Fig. 2. Effect of ALA treatment and its combination with metformin on serum total cholesterol levels. Data expressed as the mean ± SEM; [*]$p < 0.05$ as compared to naive, [#]$p < 0.05$ as compared to zymosan; [$]$p < 0.05$ as compared to ALA (50 mg/kg); [α]$p < 0.05$ as compared to ALA (100 mg/kg); [β]$p < 0.05$ as compared to ALA (200 mg/kg); [γ]$p < 0.05$ as compared to Met (100 mg/kg); One-way ANOVA followed by Tukey's test. ALA: Alpha lipoic acid; Met: Metformin.

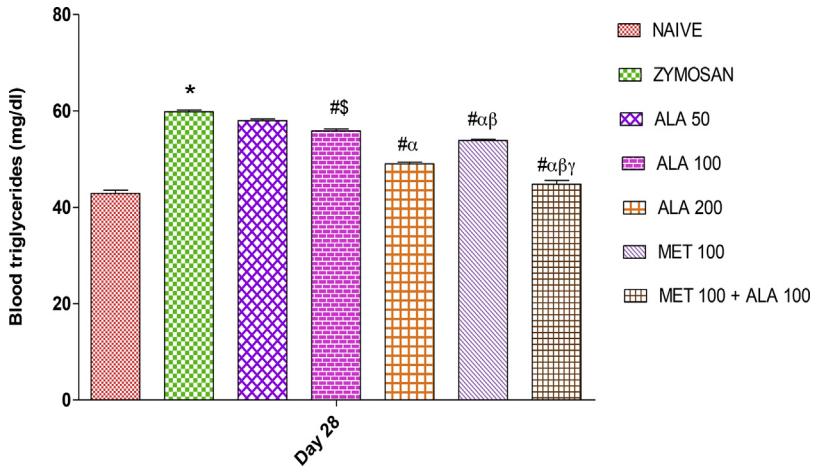

Fig. 3. Effect of ALA treatment and its combination with metformin on serum triglycerides levels. Data expressed as the mean ± SEM; [*]$p < 0.05$ as compared to naive, [#]$p < 0.05$ as compared to zymosan; [$]$p < 0.05$ as compared to ALA (50 mg/kg); [α]$p < 0.05$ as compared to ALA (100 mg/kg); [β]$p < 0.05$ as compared to ALA (200 mg/kg); [γ]$p < 0.05$ as compared to Met (100 mg/kg); One-way ANOVA followed by Tukey's test. ALA: Alpha lipoic acid; Met: Metformin.

ALA (50 mg/kg, 100 mg/kg and 200 mg/kg) and metformin (100 mg/kg) significantly decreased the insulin levels and HOMA-IR as compared to zymosan treated group, with ALA (200 mg/kg) producing most significant effect. However, treatment with [Met (100) + ALA (100)] combination significantly decreased the insulin levels and HOMA-IR as compared to zymosan group and their effects *per se* (Table 3).

Effect of ALA treatment and its combination with metformin on HbA1c levels

Blood HbA1c level in zymosan group was approximately 3-fold higher than in naïve group, however ALA treatment (50 mg/kg, 100 mg/kg and 200 mg/kg) and metformin (100 mg/kg) significantly lowered the blood HbA1c, with ALA (200 mg/kg) producing

Table 3
Effect of ALA treatment and its combination with metformin on insulin levels and HOMA-IR.

Treatment (mg/kg)	Insulin (μU/ml)	HOMA-IR
Naive	10.3 ± 0.2	2.5 ± 0.05
Zymosan	16.6 ± 0.35[a]	6.7 ± 0.17 [a]
ALA (50)	15.3 ± 0.09 [b]	5.6 ± 0.10 [b]
ALA (100)	14.6 ± 0.15 [b]	4.9 ± 0.04 [b,c,d]
ALA (200)	12.5 ± 0.21 [b,c,d]	3.8 ± 0.09 [b,c,d]
Met (100)	13.7 ± 0.20 [b,e]	4.4 ± 0.10 [b,e]
Met (100) + ALA (100)	11.7 ± 0.21 [b,d,f]	3.5 ± 0.01 [b, d,f]

All the data were expressed as the mean ± SEM; [a]$p < 0.05$ as compared to naive, [b]$p < 0.05$ as compared to zymosan; [c]$p < 0.05$ as compared to ALA (50 mg/kg); [d]$p < 0.05$ as compared to ALA (100 mg/kg); [e]$p < 0.05$ as compared to ALA (200 mg/kg); [f]$p < 0.05$ as compared to Met (100 mg/kg); One-way ANOVA followed by Tukey's test. ALA: Alpha lipoic acid; Met: Metformin.

most significant effect. Combination treatment of [Met (100) + ALA (100)] significantly improved the serum HbA1c levels as compared to zymosan group and their effects *per se* in CRS mice (Fig. 4).

Effect of ALA treatment and its combination with metformin on serum corticosterone levels

`Zymosan treated mice showed marked increase in serum corticosterone as compared naive mice. ALA treatment (50 mg/kg, 100 mg/kg and 200 mg/kg) and metformin (100 mg/kg) were found to significantly decrease the corticosterone levels as compared to zymosan group. Combination treatment [Met (100) + ALA (100)] produced significant effect as compared to zymosan group and their effects *per se* (Fig. 5).

Effect of ALA treatment and its combination with metformin on levels of serum adiponectin and IL-6

The levels of the insulin-sensitizing adipokine, adiponectin were significantly reduced in zymosan treated group as compared to naïve group. Treatment with ALA (100 mg/kg and 200 mg/kg) and metformin (100 mg/kg) significantly increased adiponectin levels. Moreover, treatment with (Met (100) + ALA (100)) combination significantly increased adiponectin levels compared to zymosan group and their effects *per se*. Serum IL-6 levels were significantly raised in zymosan treated animals as compared to naïve group. Treatment with ALA (50 mg/kg, 100 mg/kg and 200 mg/kg) and metformin (100 mg/kg) caused a significant reduction in the levels of IL-6 with ALA 200 mg/kg having most significant effect. Treatment with [Met (100) + ALA (100)] combination reduced the IL-6 levels significantly as compared to zymosan group and their effects *per se* (Table 4).

Effect of ALA treatment and its combination with metformin on zymosan induced cognitive deficit

The escape latency during the learning trial significantly decreased in all the treatment groups from day 1 to day 4 compared to zymosan treated group. Zymosan group showed significantly higher and constant latency on all 4 days of learning

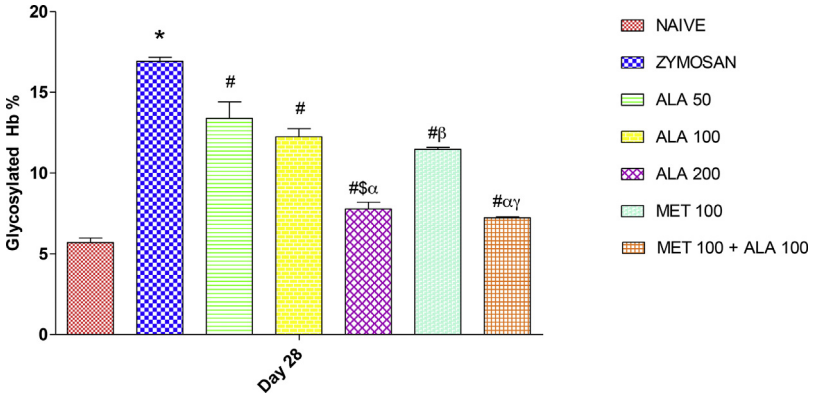

Fig. 4. Effect of ALA treatment and its combination with metformin on HbA1c levels. Data expressed as the mean ± SEM; [*]$p < 0.05$ as compared to naive, [#]$p < 0.05$ as compared to zymosan; [$]$p < 0.05$ as compared to ALA (50 mg/kg); [α]$p < 0.05$ as compared to ALA (100 mg/kg); [β]$p < 0.05$ as compared to ALA (200 mg/kg); [γ]$p < 0.05$ as compared to Met (100 mg/kg); One-way ANOVA followed by Tukey's test. ALA: Alpha lipoic acid; Met: Metformin.

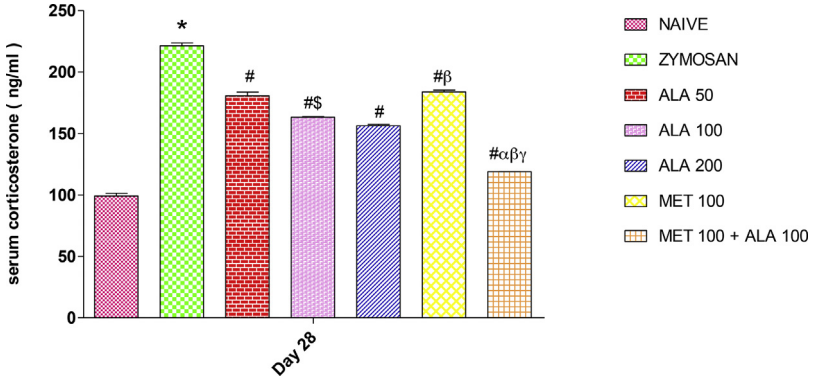

Fig. 5. Effect of ALA treatment and its combination with metformin on serum corticosterone levels. Data expressed as the mean ± SEM; [*]$p < 0.05$ as compared to naive, [#]$p < 0.05$ as compared to zymosan; [$]$p < 0.05$ as compared to ALA (50 mg/kg); [α]$p < 0.05$ as compared to ALA (100 mg/kg); [β]$p < 0.05$ as compared to ALA (200 mg/kg); [γ]$p < 0.05$ as compared to Met (100 mg/kg); One-way ANOVA followed by Tukey's test. ALA: Alpha lipoic acid; Met: Metformin.

Table 4
Effect of ALA treatment and its combination with metformin on adiponectin and IL-6.

Treatment (mg/kg)	Adiponectin (μg/ml)	IL-6 (pg/ml)
Naive	22.29 ± 1.00	52.7 ± 1.48
Zymosan	9.5 ± 1.00 [a]	202.1 ± 1.45 [a]
ALA (50)	12.8 ± 0.58	163.0 ± 1.5 [b]
ALA (100)	15.4 ± 1.22[b]	111.88 ± 1.12 [bc]
ALA (200)	18.11 ± 0.58[b,c]	71.0 ± 0.60 [b,d]
Met (100)	16.81 ± 0.25[b]	104.3 ± 1.30 [b,d,e]
Met (100) + ALA (100)	20.7 ± 0.9[b,d,f]	60.65 ± 0.35 [b,d,e,f]

All the data were expressed as the mean ± SEM; [a]$p < 0.05$ as compared to naive, [b]$p < 0.05$ as compared to zymosan; [c]$p < 0.05$ as compared to ALA (50 mg/kg); [d]$p < 0.05$ as compared to ALA (100 mg/kg); [e]$p < 0.05$ as compared to ALA (200 mg/kg); [f]$p < 0.05$ as compared to Met (100 mg/kg); One-way ANOVA followed by Tukey's test. ALA: Alpha lipoic acid; Met: Metformin.

trial as compared to naïve group (Fig.6. (a)). In addition, zymosan treatment significantly decreased the number of entries and the time spent in the target quadrant as compared to naïve group (Fig. 6(b) and (c)). However, treatment with all the doses of ALA (50 mg/kg, 100 mg/kg and 200 mg/kg) and metformin (100 mg/kg) demonstrated significant decrease in latency, increase in the number of entries and the time spent in the target quadrant compared to zymosan group. Combination treatment (Met (100) + ALA (100)) also showed significant decrease in escape latency and increase in the time spent in target quadrant and number of entries in target quadrant as compared to zymosan treated group and their effects *per se*. However no significant differences were found in swim speed among all the groups (results not shown).

Effect of ALA treatment and its combination with metformin on oxidative stress parameters in mouse hippocampus, cortex and liver

Zymosan treatment produced significant oxidative damage in mouse hippocampus, cortex and liver evident by increase in levels of MDA and nitrite, and reduction in GSH and catalase levels as compared to naive group. However, ALA (50 mg/kg, 100 mg/kg and 200 mg/kg) treatment attenuated oxidative damage evident by significant change in all the parameters of oxidative stress as compared to zymosan group. Treatment with (Met (100) + ALA (100)) significantly decreased MDA, nitrite levels and increased GSH and catalase levels as compared to zymosan group and their effects *per se* in (Tables 5 and 6).

Effect of ALA treatment and its combination with metformin on acetylcholinesterase enzyme activity in mice brain

An elevated acetylcholinesterase enzyme activity was observed in hippocampus and cortex of zymosan group, which was significant as compared to naïve group. Treatment with ALA (50 mg/kg, 100 mg/kg and 200 mg/kg) dose dependently attenuated the increased levels of acetylcholinesterase in both hippocampus and cortex which was significant as compared to zymosan group [Fig. 7(a) and (b)]. Treatment with [Met (100) + ALA (100)] significantly lowered the cortical acetylcholinesterase levels as compared to zymosan group and their effects *per se* but no potentiating effect of combination group was observed in hippocampal acetylcholinesterase levels.

Discussion

Decreased response of the peripheral tissues to insulin action, defined as insulin resistance is regarded as a hallmark of T2DM [37]. When insulin binds to its receptor, it leads to tyrosine phosphorylation of the receptor itself. Auto-phosphorylation on tyrosine residues of beta subunit further stimulates phosphorylation of insulin

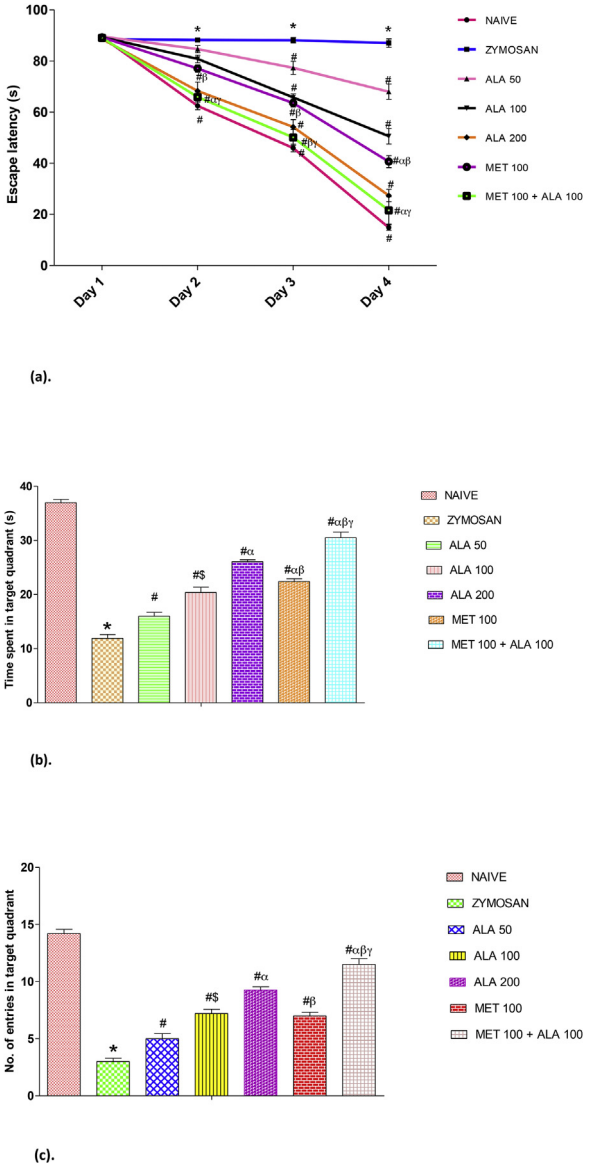

Fig. 6. Effect of ALA treatment and its combination with metformin on zymosan induced cognitive deficit (a) escape latency (b) time spent in target quadrant (c) number of entries in target quadrant. Data expressed as the mean ± SEM; [*]$p < 0.05$ as compared to naive, [#]$p < 0.05$ as compared to zymosan; [$]$p < 0.05$ as compared to ALA (50 mg/kg); [α]$p < 0.05$ as compared to ALA (100 mg/kg); [β]$p < 0.05$ as compared to ALA (200 mg/kg); [γ]$p < 0.05$ as compared to Met (100 mg/kg); One-way ANOVA followed by Tukey's test. ALA: Alpha lipoic acid; Met: Metformin.

receptor substrate (IRS) 1, 2, 3, 4 at tyrosine residues. Activated IRS-1 or IRS-2 in turn activate insulin signalling pathways such as phosphatidylinositol (PI) 3-kinase and AKT/PKB [38]. Whereas phosphorylation of IRS-1 at serine by proinflammatory cytokines leads to the impairment of insulin signalling finally leading to a state of metabolic dysfunction known as insulin resistance. Many factors like diet, obesity and stress influences the action of insulin and all of them culminates at a common pathway i.e. inflammation induced insulin resistance which is a strong risk factor for the development of T2DM.

TLRs are the innate immune cell receptors that play an important role in the activation of immune system, inflammation in obesity and in the recognition of damage-associated molecular patterns (DAMPs), during type 2 diabetes. Their activation leads to several actions like phagocytosis [39], cytokine production [40]

Table 5
Effect of ALA treatment and its combination with metformin on LPO and GSH in mouse hippocampus and cortex.

Treatment (mg/kg)	LPO moles of MDA/mg protein (% naive)		GSH μmoles of GSH/ mg protein (% naive)	
	Hippocampus	Cortex	Hippocampus	Cortex
Naive	1.2 ± 0.02	1.1 ± 0.02	0.01 ± 0.0001	0.014 ± 0.0005
Zymosan	4.4 ± 0.03 [a]	4.6 ± 0.03 [a]	0.0014 ± 0.0004 [a]	0.0019 ± 0.0001 [a]
ALA (50)	3.4 ± 0.02 [b]	3.9 ± 0.002[b]	0.002 ± 0.0002	0.0028 ± 0.0001
ALA (100)	2.7 ± 0.04 [b,c]	3.8 ± 0.06 [b]	0.003 ± 0.0001 [b]	0.004 ± 0.0004[b]
`ALA (200)	2.4 ± 0.03 [b,d]	2.2 ± 0.003 [b,d]	0.005 ± 0.0007 [b]	0.005 ± 0.0002[b]
Met (100)	2.4 ± 0.02 [b,d]	2.5 ± 0.02[b, e]	0.003 ± 0.0004 [b,e]	0.003 ± 0.0001[b,e]
Met (100) + ALA (100)	1.2 ± 0.01[b,d,e,f]	1.3 ± 0.04[b,d,e, f]	0.008 ± 0.0006[b,d,e, f]	0.007 ± 0.0004[b,d,e,f]

All the data were expressed as the mean ± SEM; [a]$p < 0.05$ as compared to naive, [b]$p < 0.05$ as compared to zymosan; [c]$p < 0.05$ as compared to ALA (50 mg/kg); [d]$p < 0.05$ as compared to ALA (100 mg/kg); [e]$p < 0.05$ as compared to ALA (200 mg/kg); [f]$p < 0.05$ as compared to Met (100 mg/kg); One-way ANOVA followed by Tukey's test. ALA: Alpha lipoic acid; Met: Metformin.

Table 6
Effect of ALA treatment and its combination with metformin on catalase and nitrite in mouse hippocampus and cortex.

Treatment (mg/kg)	Catalase μmoles of H_2O_2/min/mg protein (% naive)		Nitrite mg/ml (% naive)	
	Hippocampus	Cortex	Hippocampus	Cortex
Naive	0.8 ± 0.01	1.0 ± 0.025	620.4 ± 1.81	640.4 ± 1.52
Zymosan	0.2 ± 0.002[a]	0.2 ± 0.004 [a]	1235.5 ± 2.72 [a]	1306.5 ± 2.69 [a]
ALA (50)	0.3 ± 0.04	0.4 ± 0.02[b]	1060.6 ± 0.77 [b]	1143.5 ± 5.50 [b]
ALA (100)	0.5 ± 0.034[b,c]	0.6 ± 0.03 [b,c]	1066.1 ± 1.05 [b]	1139.1 ± 1.71 [b]
ALA (200)	0.7 ± 0.04 [b,d]	0.8 ± 0.05 [b,d]	797.6 ± 2.39 [b,d]	884.5 ± 0.92 [b,d]
Met (100)	0.06 ± 0.008 [b,d]	0.7 ± 0.04[b]	845.1 ± 0.39[b,d,e]	1033.6 ± 0.32[b,d,e]
Met (100) + ALA (100)	0.2 ± 0.006 [d]	0.9 ± 0.006 [b,d,f]	630.7 ± 0.44[b,d,f]	677.7 ± 0.58 [b,d,f]

All the data were expressed as the mean ± SEM; [a]$p < 0.05$ as compared to naive, [b]$p < 0.05$ as compared to zymosan; [c]$p < 0.05$ as compared to ALA (50 mg/kg); [d]$p < 0.05$ as compared to ALA (100 mg/kg); [e]$p < 0.05$ as compared to ALA (200 mg/kg); [f]$p < 0.05$ as compared to Met (100 mg/kg); One-way ANOVA followed by Tukey's test. ALA: Alpha lipoic acid; Met: Metformin.

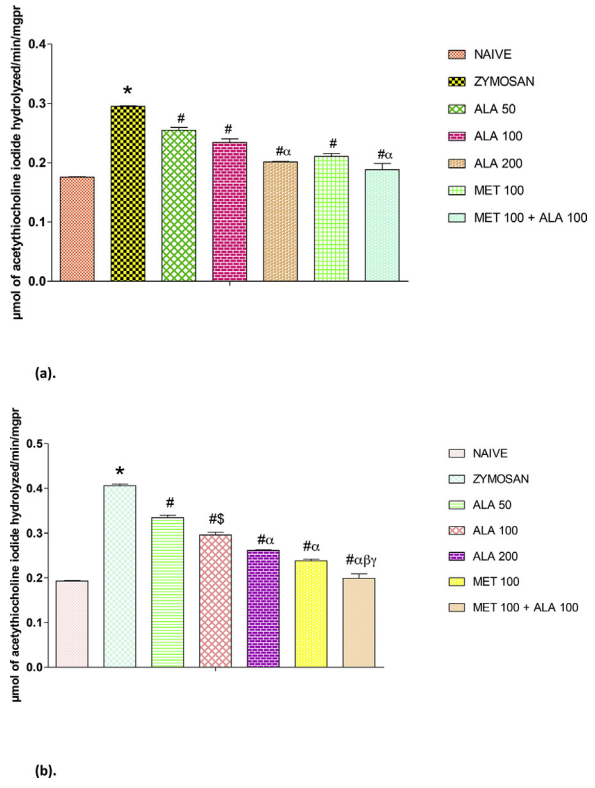

Fig. 7. Effect of ALA treatment and its combination with metformin on brain acetylcholinesterase enzyme activity of mice (a) hippocampal (b) cortical. Data expressed as the mean ± SEM; [*]$p < 0.05$ as compared to naive, [#]$p < 0.05$ as compared to zymosan; [$]$p < 0.05$ as compared to ALA (50 mg/kg); [α]$p < 0.05$ as compared to ALA (100 mg/kg); [β]$p < 0.05$ as compared to ALA (200 mg/kg); [γ]$p < 0.05$ as compared to Met (100 mg/kg); One-way ANOVA followed by Tukey's test. ALA: Alpha lipoic acid; Met: Metformin.

and expression of co-stimulatory molecules and adhesion molecules [41,42]. Numerous studies demonstrate that TLR2 contributes to the development of inflammation induced insulin resistance through activation by exogenous ligands like dietary fatty acids and enteric lipopolysaccharides and endogenous ligands [43].

Zymosan, a toll like receptor 2 agonist, is a mixture of cell-wall particles from the yeast named *Saccharomyces cerevisiae* [43,44]. When it was injected into mice once in a week for 28 days in the present study, it produced marked inflammatory response by binding to TLR2 that resulted in functional and biochemical alterations in remote organs, including liver and brain. Upon binding to zymosan, TLR2 leads to increased expression of TLR and stimulation of MyD88-dependent signalling culminating in NF-kB transactivation, leading to significant proinflammatory cytokine production [45]. These released inflammatory mediators lead to serine phosphorylation of IRS-1 [46], as opposed to tyrosine phosphorylation [47], inhibiting insulin signaling. JNK and IKKβ/NF-κB are also activated by the toll like receptors [48]. This was evident in the present study by significant rise in the inflammatory marker IL-6 and decrease in anti-inflammatory cytokine adiponectin. The systemic inflammatory response produced by zymosan produced both metabolic and hormonal changes, evident by increased basal levels of stress hormone corticosterone [49] and IR which is consistent with previous reports [14]. The successful development of insulin resistance by zymosan was supported by the following findings: hyperglycaemia, hyperinsulinemia, hyperlipidaemia, hypoadiponectinemia, increase in glycosylated haemoglobin and increased HOMA-IR values which is the widely used insulin sensitivity marker. Moreover it led to significant increase in body weight when measured on day 14, 21 and 28, as compared to naive control. It has been reported that cytokine induced inflammation leads to adipogenesis, a factor for weight gain [50]. The other reason for it is that increased insulin during insulin resistance causes lipogenesis which also contributes to weight gain.

Chronic administration of ALA demonstrated dose dependent effect in ameliorating insulin resistance in zymosan treated mice with highest dose of ALA showing most significant effect. The protective effect of ALA has been concluded from its potential to reduce body weight, plasma glucose, insulin levels, HOMA-IR, Hb1Ac, serum cholesterol and TGs. Standard drug metformin also decreased the level of stress hormone corticosterone and ameliorated all the parameters of insulin resistance, but it was found less effective than the highest dose of ALA. However, co-administration of ALA with metformin caused significant reduction in elevated glucose levels, plasma insulin levels, HOMA-IR, Hb1ac levels, triglycerides and cholesterol levels as compared to zymosan treated group and their effects *per se* thus showing potentiating effect.

ALA administration in the present study produced anti-inflammatory effect characterised by decreased serum IL-6 levels and significant increase in circulating levels of adiponectin. The ALA is reported to inhibit IκB degradation and NF-κB-dependent gene expression [51]. Thus we can hypothesise that stimulation of MyD88 dependent signalling by zymosan, after binding TLR2 receptor that culminates in NFκβ transactivation is inhibited by ALA. This in turn will lead to decreased production of inflammatory cytokines and improved insulin sensitivity. Moreover, ALA have been reported to decrease the gene expression of TLR2 [21] which has role in insulin resistance [22]. Our results also showed anti-inflammatory effect of metformin along with improved insulin sensitivity [52].

Activation of TLR2 resulted in oxidative stress in brain and liver in the present study. A similar result reported that activation of TLR2, TLR3, and TLR4 produced pro-oxidative effects on intestinal epithelial cells [53]. Whereas daily administration of ALA produced marked antioxidant effects by significantly increasing the levels of anti-oxidant defence system and reducing the levels of MDA and nitrites. ALA has been reported to enhance intracellular glutathione levels by acting as inducer of genes governing GSH synthesis [54], besides inhibiting NF-κB induced ROS generation. Similar antioxidant effect was found with metformin but less than the highest dose of ALA. Previous study also reports antioxidant effect of metformin [55].

Zymosan administration resulted in the decline in learning and memory, the results of which were evident from the morris maze test. As previous and recent findings suggest the role of toll like receptors in the Alzheimer's disease process, so this effect of zymosan may be attributed to the inflammation caused by it after binding to TLR2 in brain [56–59]. Studies indicate that innate inflammatory immune response is a powerful pathogenic response in the neurodegeneration process as well. TLRs activation occur in response to infectious agents, tissue injury or autoimmune conditions in glial cells (microglia, astrocytes and oligodendrocytes) and lymphocytes that infiltrate the nervous system [60]. This in turn stimulates the production of pro-inflammatory cytokines and cell adhesion molecules in immune cells, indirectly damaging neurons and leading to decline in cognitive function. Along with the direct action of zymosan in brain, impaired insulin signalling in brain caused by zymosan may also be the contributing factor in decline of cognitive function. Elevated levels of corticosterone after zymosan injection may also contribute to deregulated hippocampal functioning [61], leading to distorted spatial memory [62] correlated to stress. Same was confirmed by morris water maze. Acetylcholine is a neurotransmitter in brain found to have a role in cognition and memory [63], which is being degraded by the enzyme acetycholinesterase. Neuroinflammation was reported to induce acetycholinesterase activity [64]. In our study also there is parallel rise in actylcholinesterase activity with elevated levels of proinflammatory cytokines. ALA can readily cross blood brain barrier [65] and normalizes the acetylcholinesterase activity [66], Moreover it is reported to decrease corticosterone level and IR, hence treatment of zymosan injected mice with ALA demonstrated significant improvement in cognitive function along with decreased oxidative stress and acetylcholinesterase enzyme activity in hippocampus and cortex [67]. Combination treatment with Met 100 + ALA 100 showed potentiating effect in ameliorating insulin resistance and cognitive deficit produced by TLR2 activation.

Conclusion

In conclusion, ALA ameliorated insulin resistance and cognitive decline induced by zymosan by inhibiting oxidative stress and inflammatory pathways activated by TLR2. Study further provided scientific evidence that ALA could be used as adjunct drug along with other anti-diabetic drugs in the management of insulin resistance and cognitive deficit associated with T2DM.

Conflict of interest

The authors declare no conflict of interest.

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