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Cellular investigations to uncover curative potentials of polyphenols- An *in vitro* study of Apple Cider Vinegar (ACV) and Chrysin against Alzheimer's like pathology via down-regulation of AChE activity

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Amyloid aggregation and neurofibrillary pathology is the characteristic feature of Alzheimer's disease (AD). Streptozotocin (STZ) is a glucosamine nitrosourea compound that is toxic to the cells, impairs insulin signaling in the brain. STZ induces DNA damage and oxidative stress, which leads to cognitive impairments. In this experimental study, STZ treatment induces Alzheimer's pathology in mouse neuroblastoma (N2A) cells. The study also explored the protection of cellular damage by pre-treatment with test drugs Apple cider vinegar (ACV), chrysin and rivastigmine. This study had been concentrated mainly on the cellular mechanism of neuromodulation and anti –oxidant potency of test drugs to attenuate cellular toxicity induced by STZ treatment. The 100 µM concentration of STZ was used for treatment in N2A cells for 24 h and 48 h and multiple studies were performed. The STZ causes tau phosphorylation, amyloid aggregation and increased acetyl cholinesterase (AChE) activity. Along with STZ at a concentration of 100 µM, the cells were pre-treated with ACV, chrysin and rivastigmine at a concentration of 2, 10, 50 µM each. The results show that 2 µM test drug concentration presented considerable protection, against STZ generated neurocytotoxicity via. restoration of anti-oxidant enzymes, MDA and AChE level, compared to the other two concentrations.

Keywords: AChE activity, Amyloid aggregation, Oxidative stress markers, Streptozotocin, Tauopathy

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In the 20th century, unprecedented work pressure and a fast-pacing urban lifestyle made severe diseases, like diabetes, cardiovascular complications, respiratory illness, neurological disorders and cancer a widespread phenomenon. Cognitive impairments and loss of thinking ability, impaired neuronal communication and memory loss are the most common features of dementia. These prolonged symptoms collectively produced a loss of function and development of neuro defects in the geriatric population and are commonly referred to as Alzheimer's disease (AD).

AD is a progressive neurodegenerative disorder characterized by psychological disabilities along with motor incoordination. However, in some families, AD is inherited as an autosomal dominant disorder of midlife. Usually, AD is recognized in the later stage when cognitive impairment appears[1]. In the literature, numerous models have been anticipated to explain tools and pathways regarding AD initiation. AD pathology included loss of cholinergic function, intracellular tau protein (microtubule-associated protein) hyperphosphorylation and extracellular β-amyloid plaque formation[2]. AD faces two main challenges in the present scenario: delay in the diagnosis process and subsequently lack of target neuroprotective agent or curative pharmacological treatment.

The formation of extracellular amyloid protein aggregation is the primary pathological indication of Alzheimer's pathology. In the brain, insulin is synthesized locally and transported via., a blood-brain barrier to peripheral circulation[3]. So the interruption in insulin signaling leads to learning and memory impairments[4]. Current reports supported the assumption that impaired brain insulin signaling, deregulation in energy and glucose utilization are important pathogenic causes of amyloid precursor protein (APP) processing[5]. Amyloid protein is the portion of the APP protein. When β and γ-secretase enzymes split APP protein unevenly, it produces the excessive formation of amyloid protein that gets aggregated extracellularly[6].

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Like amyloid aggregation, tauopathy is another pathological indicator of AD. Microtubules associated protein tau gets hyperphosphorylated in the tauopathy condition and forms coiled paired helical filament (PHF) structures, known as neurofibrillary tangles (NFTs). These tangles are then accumulated intracellularly in the Alzheimer's brain[7,8].

These hallmarks, amyloid aggregation and tauopathy, both reduce the acetylcholine (ACh) neurotransmitter level considerably in AD. ACh is the leading neurotransmitter of the Central Nervous System (CNS), which plays a dynamic role in cognitive activity. During AD pathology, the ACh level drives downwards and ACh associated enzymatic activity of acetylcholinesterase (AChE) is enhanced cause the role of AChE is to break down the ACh into choline and acetate compound. Further, choline is re-uptaken by nerve terminals and reused for the synthesis of ACh neurotransmitters. The increased AChE activity demonstrated memory alteration and AD-like symptoms[9].

Coming to AD treatment, various medications are present globally, but they are short of being called a successful treatment against AD. Their benefits may vary from person to person, and there are many arguments among the researchers about the efficacy of current medications. The earlier animal studies have shown that polyphenols are effective against AD-like symptoms[8]. Therefore, we strive to confirm the effectiveness of polyphenols further by carrying out studies at the cellular level.

In nature, flavonoids are the natural polyphenols present in plants and contain various pharmacological activities like anti-inflammatory, antiviral, anti-tumor and anti-oxidant activity[10,11]. Chrysin is a flavonoid compound found in honey and various plant extracts. Chrysin has reported various pharmacological activities like anti-allergic, antidiabetic, anticancer, anti-inflammatory and anti-oxidant properties[11–15].

Similarly, apple juice is one of the most consumed fruit juice in daily life. Apple Cider Vinegar (ACV) is produced from a cider that has undergone bioconversion procedures. It contains acetic acid, organic acids, flavonoids, polyphenols, vitamins and minerals but phenolic compounds are higher in concentration[8,16,17]. ACV comprises beneficial health effects for cardiovascular diseases like hyperlipidemia, hypercholesterolemia, diabetes, cancer and obesity[17–20].

Several experimental models using transgenic and non-transgenic animals have been used to study AD's dynamic outcomes[21,22]. In the rodent model, STZ administration via., an intracerebroventricular (ICV) route in the rat brain induces Alzheimer's like pathology[23]. Non-transgenic models consist of STZ[23], β-amyloid[24] and okadaic acid[25] induced disease AD model on experimental rodents. So, the detailed effects of STZ on neuronal (N2A) cells is a novel experimental model for AD-like pathological markers like oxidative stress, tauopathy condition, amyloid aggregation, disrupted glucose uptake neuroinflammation, glial cell activation and deficiency in cholinergic activity.

The current study was focused on the STZ-prompted oxidative stress on N2A cells. Therefore, the present study is designed to investigate STZ-prompted *in vitro* neurotoxicity on N2A cells and its consequent protection with chrysin, ACV and rivastigmine.

Methodology

The cell line work was performed in the Indian Institute of Technology (IIT), Kanpur from March to May 2019. In the *in vitro* experiment analysis, multiple duplicates of culture cells were prepared via., pipetting the equal volume of the cells. These culture samples were made from the same culture stock of cells. All the experiments had triplicate cultures and every experiment was performed three independent times.

Material and Methods

Chemicals required

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, Streptomycin-penicillin antibiotic solution, Dulbecco's modified Eagle's medium (DMEM), laboratory chemical like formaldehyde, Triton-X 100, streptozotocin (STZ), glutathione reductase, Nicotinamide Adenine dinucleotide phosphate (NADPH), Nicotinamide Adenine dinucleotide (NADH), ethylenediaminetetraacetic acid (EDTA), Xanthine oxidase (XOD), Thiobarbituric acid (TBA), Nitro-blue tetrazolium chloride (NBT), Trichloroacetic acid (TCA), Congo red, thioflavin-S dye 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), 4',6-diamidino-2-phenylindole dihydro chloride (DAPI), DPX (Dibutylphthalate Polystyrene Xylene), Acetylthiocholine iodide, chrysin and rivastigmine were procured from Sigma-Aldrich, USA. ACV was obtained from the manufacturing sources (The Unati Co-op Marketing-cum-processing Society Ltd., an ISO 22000:2005 certified company, Punjab).

Cell culture & treatment

Originally, Neuro2A (N2A) cells are derived from albino mouse brains, which reveal similar neuronal morphology characters, unlike other neuroblastoma cells[26]. This cell line was obtained from National Centre for Cell Sciences (NCCS) Pune, India and cultured in DMEM media with 10% fetal bovine serum (FBS) solution and 1% antibiotic solution. These cell lines were preserved in a humidified atmosphere (5% CO_2 and 95% air) at 37°C. Once they reached 85% confluence, then they were used for further experiments.

STZ was used to disrupt the insulin signaling in brain, leading to alteration of oxidative stress markers, induce amyloid pathogenesis and tauopathy[27]. So, in the current study, 100 µM concentration of STZ was used in N2A cells for 24 and 48 h to induced cellular toxicity and co-treatment with ACV, chrysin and rivastigmine at 2, 10, 50 µM concentrations (1 h before addition of STZ) were given to evaluate their effect on STZ induced cellular toxicity.

Cell viability assay (MTT)

The cell viability assay was conducted by using MTT dye to evaluate the cellular toxicity on the cells. The assay principle is to form violet formazan crystals in the viable cells by the division of tetrazolium salts in the presence of mitochondrial succinate reductase enzyme[28]. The N2A cell culture (1×10^6) containing DMEM media was seeded into a 96-well plate. After test drug treatments, the plate was placed within an incubator (37°C) for 24 and 48 h. After 24 and 48 h, MTT dye (0.5 mg/mL) solution was added to each well of the 96-well plate and plates were again incubated for 4 h. The supernatant was then discarded and 150 µL of fresh filtered DMSO was added to solubilize the formazan crystals. The color intensity was measured using a Spectra Max-3 spectrophotometer plate reader at a wavelength of 570 nm and calculation was performed via the following formula.

$$Percentage\ of\ cell\ viability = \frac{(Abs\ test - Abs\ blank)}{(Abs\ control - Abs\ blank)} \times 100$$

Where, Abs = absorbance

Estimation of Alzheimer's like pathological markers

Thioflavin-S staining of cells (tauopathy)

Thioflavin-S is a benzothiazole dye used to visualize hyperphosphorylated tau protein aggregation in both *in vivo* and *in vitro* experiments[29]. In the staining process, cells (1×10^6) were developed on gelatin-coated coverslips then exposed to STZ treatment at 100 µM concentration and test drug treatment at a 2 µM concentration. After 24 h incubation period, 4% formaldehyde solution was used to fix the cells on coverslips and washed once with PBS solution at room temperature. A solution of 0.05% thioflavin-S dye in 50% ethanol was prepared to stain the cells. After staining the cells, three successive washes of 70% ethanol (5 min each), was given to the cells. At last, cells were counter stained with DAPI and mounted with glycerol to observe under a Y-TV55 Nikon fluorescence microscope.

Congo red staining of cells (amyloid aggregation)

Congo red dye was used for the validation of amyloid aggregation via bind with the Aβ plaques. So, this procedure was performed to detect extracellular amyloid plaque accumulation in the N2A cells[30]. In this procedure, the N2A cells (1×10^6) were developed on gelatin-coated coverslips and exposed to 100 µM concentration of STZ and 2 µM concentration of test drugs. After 24 h of the incubation period, 4% (v/v) formaldehyde solution was used to fix the cells on coverslips at room temperature and then the cells were washed one time with PBS solution. The alkaline Congo red solution was freshly prepared to stain the cells at room temperature. After staining, the cells were washed with deionized water (three washes) and mounted in DPX to observe under a Leica bright-field microscope.

Anti-oxidant activity

Reduced glutathione assay (GSH)

Reduced GSH is an endogenous anti-oxidant, which is made during oxidative stress conditions. GSH enzyme protects against endogenous reactive oxygen species (ROS). GSH was determined by using an enzymatic method of Tietze *et al.*[31] with certain modifications. Cells (1×10^6) were seeded on 30 mm Petri plates and incubated overnight before any treatment in this assay. After the incubation period, cells were washed with PBS solution and treated with 10% trichloroacetic acid (TCA) solution. This cell mixture was centrifuged at 13,000 rpm for 1 min. To estimate the GSH level in the N2A cells, final 1 mL reaction mixture volume contained 50 µL sample (supernatant), 0.1 M sodium phosphate buffer (pH 7.4), 1 unit/mL glutathione reductase, 5 mM EDTA solution, 0.6 mM DTNB and 0.2 mM β-NADPH solution. The absorbance was noted at

412 nm and the following formula was used to calculate the GSH level.

$$Reduced\ GSH\ Concentration = \frac{Abs\ of\ the\ test - Abs\ of\ blank}{6.22 \times 0.1} \times total\ volume\ of\ assay$$

Where, 1= volume of the assay mixture

6.22= Millimolar extinction coefficient of β-NADPH

0.1= enzyme volume

Lipid peroxidation level (MDA)

Oxidative stress leads to protein, lipid peroxidation and the aging process. MDA is the biomarker of lipid peroxidation. So any high increase in MDA level indicates low efficacy of the drug. MDA level was conducted by the process of Ohkawa *et al.*[33]. N2A cells were cultured on 30 mm Petri plates with 1×10^6 cells/mL concentration and were treated with STZ (100 µM) and test drugs with 2, 10 µM concentration. After the subjective treatment of test drugs, cells were incubated for 24 h. The cells were then washed with PBS solution and lysed in 1% KCl with 1% Triton X-100 solution by sonication for 5 min. Then 100 µL of cell lysate solution was taken and mixed with 200 µL of 8.1% SDS solution, 1.5 mL of 20% acetic acid (pH 3.5), 1.5 mL of 0.8% thiobarbituric acid (TBA) solution, and final 4 mL volume were made using deionized water. The reaction mixture was boiled on a water bath for 2 h for color development (pink color), followed by the cooling process. This mixture was centrifuged at 3000 rpm (10 min) and the supernatant absorbance was read at 532 nm. The MDA content was calculated using the following formula[34].

$$MDA\ concentration = \frac{Abs\ at\ 532nm}{1.56 \times 10^5}$$

Where, 1.56×10^5= molar extinction coefficient in $M^{-1}\ cm^{-1}$

Superoxide dismutase assay (SOD)

SOD enzymatically scavenges superoxide generated by oxidative stress. So SODs constitute the first line of defense against ROS' deleterious effects in neurodegenerative disorders like Alzheimer's disease. N2A cells (1×10^6) were seeded on 30 mm Petri plates and incubated overnight before any treatment. For trypsinization of adherent cells, 0.2% (w/v) EDTA and 0.5% (w/v) trypsin solution were used. Then pellet of the cells was mixed with 500 µL of 1X cell lysis solution. It was mixed thoroughly and the process was repeated. This cell suspension solution was centrifuged for 5 min at 14000 rpm at 4°C. The supernatant was placed in a clean Eppendorf tube (1.5 mL) and stored at -80°C for further analysis. The total reaction volume of the SOD assay was 1.5 mL (540 µL reagent components and 960 µL deionized water). The reaction mixture contained 30 µL of nitroblue tetrazolium (NBT) solution, 200 µL of NADH solution and 10 µL of xanthine oxidase (XOD) solution and 300 µL cell lysate solution[35]. The reduction of NBT to blue formazan crystals was measured at 560 nm under aerobic conditions and the following formula was used to measure enzyme activity[36].

$$Percentage\ Inhibition = \frac{Abs\ of\ Control - Abs\ of\ Test}{Abs\ of\ Control} \times 100$$

Acetylcholinesterase activity (AChE)

AChE assay was assessed using the mode defined by Ellman *et al.*[37] with specific alterations. In this method, the N2A cells were seeded in 35 mm Petri plates and incubated overnight before test drug treatments. The treatment of 100 µM concentration of STZ was given in N2A cells, and co-treatment with test drugs (2,10 µM) was given 1 h before the addition of STZ and incubated for 24 h. After 24 h of incubation, cells were lysed in cold 0.1 M sodium phosphate buffer (pH 7.8) with a 0.1% Triton-X 100 solution by sonication. This cell lysed solution was centrifuged at 10,000 rpm (20 min) at 4°C and the supernatant solution was used for AChE activity. The reaction mixture contains 10 µL of cell supernatant, 2.8 mL assay buffer, 0.1 mL of DTNB (10 mM) and 20 µL of acetylthiocholine iodide (100 mM) solution were mixed well. The kinetic profile was measured at 412 nm for 2 min at 15 sec intervals using a Spectra-max-3spectrophotometer.

$$Percentage\ Inhibition = \frac{Abs\ of\ control - Abs\ of\ test}{Abs\ of\ control} \times 100$$

Statistical examination

The present study data were evaluated by using ANOVA (One-way) followed by using Tukey's Multiple Comparison Test. Graph Pad Prism version 5 software was used for three independent experiments (n=3) to analyze the data. A p-value less than 0.05 was considered statistically significant and all the data were expressed as Mean±SD.

Results

Cell viability assay (MTT)

STZ treatment showed significant ($p<0.05$) differences in mitochondrial dehydrogenase activity. The cell viability of STZ (100 µM) treated cells was found 72.4%, 64.3%, respectively, after 24 h and 48 h. Co-treatment of chrysin with STZ-treated cells showed cell viability to be 80%, 42.8% and 21.7% after 24 h and 90.7%, 32.37% and 1.66% after 48 h at 2, 10, 50 µM concentrations respectively. Similarly cell viability of ACV with STZ treatment was found to be 91.4%, 82.3% and 50.5% after 24 h and 93.3%, 77,3% and 44.5% after 48 h at 2, 10, 50 µM concentrations, respectively. Cell viability of rivastigmine with STZ-treated cells were found 82.3%, 64.7% and 39% after 24 h and 76.2%, 31.8% and 29.2% after 48 h at 2, 10, 50 µM concentrations, respectively. Cell viability assay results concluded that ACV showed more protection to N2A cells than chrysin and rivastigmine treatment (Fig. 1a, b).

Quantitative analysis of Alzheimer's related pathological markers

Thioflavin-S staining of cells

Intracellular neurofibrillary tangles (NFTs) are the critical neuropathological feature of AD. In the AD pathology, abnormally phosphorylated tau protein made paired helical filaments (PHFs), which lead to the development of NFTs. This thioflavin-S dye displays green fluorescence due to the presence of PHFs in STZ-treated N2A cells. A significant ($p<0.001$) increase in green color fluorescence was found in STZ-treated cells compared with control cells. Co-treatment with test drugs showed significant (chrysin and rivastigmine, $p<0.01$ and ACV, $p<0.05$) reduction in green color fluorescence compared with STZ-treated cells. In the test groups, ACV showed better protection of cells against STZ-induced tauopathy than the other two drugs. The observations are reported at a 2 µM concentration of test drugs with STZ (100 µM) treatment (Fig. 2a).

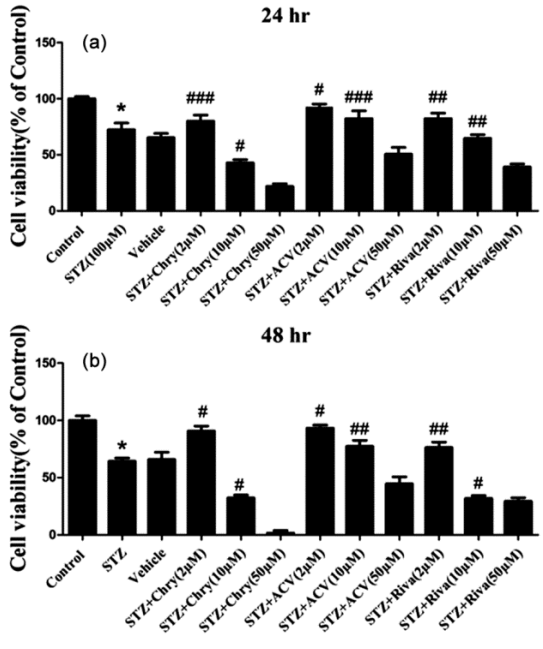

Fig. 1(a, b) — Graphical representation of Cell viability assay of STZ (100 µM) treatment and STZ+Drugs (2,10,50 µM concentrations) treatment, after 24 and 48 h. Data are represented as Mean±SD, analyzed by one-way ANOVA, Tukey's Multiple Comparison Test of three independent experiments. *p<0.05 STZ-treated cells vs. Control cells. #p<0.05, ##p<0.01, ###p<0.001 STZ+Drugs vs STZ-treated cells.

Congo red staining in cells

Congo red staining process was performed in N2A cells for the detection of the extracellular amyloid plaque development. The STZ treatment displayed a significant ($p<0.001$) increase in red-stained at 100 µM concentration compared to control cells. Co-treatment with test drugs showed significant (chrysin and rivastigmine, $p<0.05$ and ACV, $p<0.01$) reduction in red-stained compared with STZ-treated cells. Co-treatment of test drugs at 2 µM concentration offers an effective defense against STZ induced amyloid accumulation (Fig. 2b).

Assessment of anti-oxidant activity of cells

The cellular anti-oxidant activity of SOD and GSH enzymes declined significantly ($p<0.05$) in the STZ-treated group by 24.83% and 52.91%, respectively, at 100 µM concentration after 24 h as compared to control cells. Co-treatment with test drug chrysin showed a significant ($p<0.001$, $p<0.01$) increase in the SOD and GSH level as 43.35% and 74.4% after 24 h at 2 µM concentrations as compared with STZ-treated

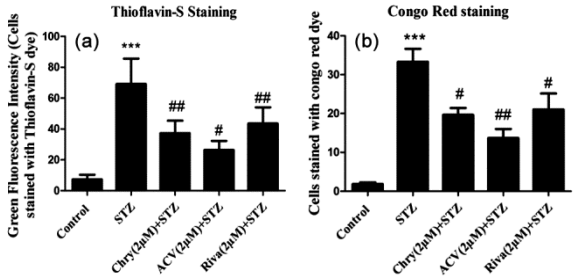

Fig. 2(a, b) — Graphical representation of Thioflavin-S (Fluorescent intensity) and Congo red-stained cells in N2A cells. Data are represented as Mean±SD, analyzed by one-way ANOVA, Tukey's Multiple Comparison Test of three independent experiments. ***p<0.001 STZ-treated cells vs. Control cells. #p<0.05, ##p<0.01 STZ+Drugs vs STZ-treated cells.

cells. Similarly, co-treatment with ACV ($p<0.05$) and rivastigmine ($p<0.01$) displayed a significant increase in the levels of SOD and GSH and the percentage increase was 68.75%, 84% for ACV and 50.47%, 76% for rivastigmine after 24 h at 2 μM concentrations as compared with STZ-treated cells (Fig. 3a,b). The results indicated a reduction in GSH level and SOD activity was improved by pre-treatment with chrysin, ACV and rivastigmine but not up to the control level.

STZ exposure in N2A cells also leads to lipids' peroxidation, which was measured by MDA level. N2A cells were exposed to 100 μM concentration of STZ showed a significant ($p<0.05$) increase in MDA level up to 232.68% after 24 h compared to control cells. Pre-treatment with chrysin, ACV and rivastigmine significantly declined in MDA level. The reduction in MDA level were as follows, for chrysin ($p<0.01$,126.82%), ACV($p<0.05$, 111.54%) and rivastigmine ($p<0.001$, 152.75%) after 24 h at 2 μM concentration as compared to STZ-treated cells (Fig. 3c).

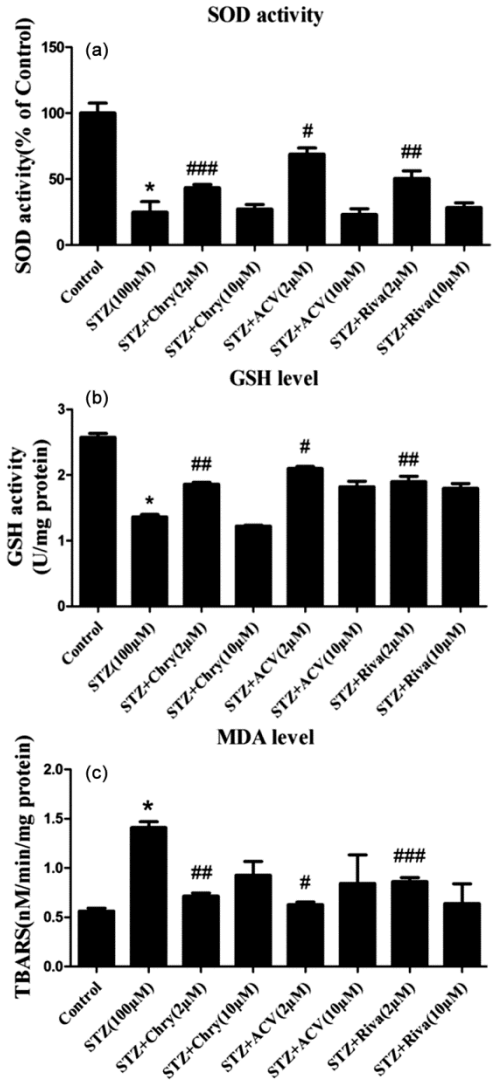

Fig. 3(a,b,c) — SOD, reduced GSH and MDA activity of STZ (100 μM) treatment and STZ+Drugs (2,10 μM concentration) treatment, after 24h. Data are represented as Mean±SD, analyzed by one-way ANOVA, Tukey's Multiple Comparison Test of three independent experiments. *p<0.05 STZ-treated cells vs. Control cells. #p<0.05, ##p<0.01, ###p<0.001 STZ+Drugs vs STZ-treated cells.

AChE activity

A significant increase ($p<0.05$) was found in the AChE activity of the STZ-treated N2A cells. The increased AChE activity was found to be 193.91% at 100 μM concentration of the STZ-treated group compared to control cells. Protection of N2A cells on pre-treatment with chrysin, ACV and rivastigmine significantly decreased AChE level, and percentage decrease was as follows for chrysin ($p<0.01$, 129.91%), ACV ($p<0.05$, 117.88%) and rivastigmine ($p<0.01$, 139.20%) respectively after 24 h at 2 μM concentration in comparison to STZ-treated cells (Fig. 4).

Discussion

The previous reports explained that STZ administration in rodents is a well-established model of dementia[23,38,39]. However, this specific action of STZ on neuronal cells was yet to be explored. Our *in vitro* exploration would provide the idea to identify Alzheimer's like physiological features at the neuronal cell level. So present study indicates the outline of STZ-induced cytotoxicity in N2A cells. In this study, dose selection of STZ was made according to previous studies, and the percentage of cell viability

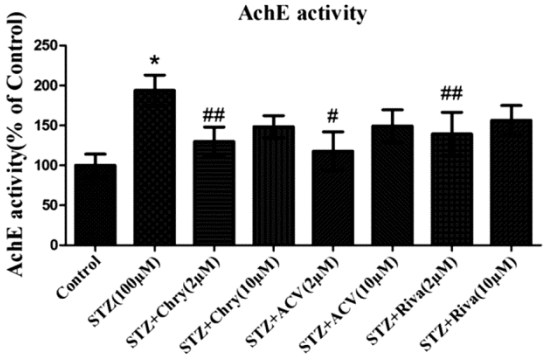

Fig. 4 — Graphical representation of AChE activity of STZ (100 μM concentration) treatment and STZ+Drugs (2,10 μM concentration) treatment, after 24 h. Data are represented as Mean±SD analyzed by one-way ANOVA, Tukey's Multiple Comparison Test of three independent experiments. *p<0.05 STZ-treated cells vs. Control cells. #p<0.05, ##p<0.01 STZ+Drugs vs STZ-treated cells.

was measured by cell viability (MTT) assay (Fig. 1a,b) after the optimum dose of STZ treatment[27].

In this study, pathological markers of AD-like tau hyperphosphorylation (tauopathy), β amyloid accumulation, AChE activity and anti-oxidant activity were assessed in the N2A cells after STZ treatment. Tau phosphorylation is the prominent indicator of AD, which was evaluated by thioflavin-S staining. Various kinase enzymes like GSK-3β and MAPK are responsible for the hyperphosphorylation of tau protein to forms NFTs[40,41]. The results indicated that STZ treatment caused a high proportion of NFTs formation in STZ-treated cells (Fig. 2a). Our results complement the finding of animal studies[42].

Further, amyloid aggregation was evaluated by Congo red staining procedure (Fig. 2b). This dye stained the extracellular protein aggregation (amyloid) present in STZ-treated neuronal cells[27]. So from the evaluation of staining results, it is clear that STZ treatment induced tauopathy and amyloid aggregation in the cells.

The STZ treatment on neuronal cells produces impaired insulin signaling and depletion of glucose utilization[43,44]. STZ treatment also creates oxidative stress estimated by anti-oxidant activity like SOD, GSH and MDA levels. The two major causes of oxidative stress are either excessive production of ROS or decreased cellular anti-oxidant levels like SOD and GSH[45]. SOD and reduced glutathione (GSH) plays a significant role in a physiological anti-oxidant and are important free radical scavenger to protect the cells. Reduction in GSH level initiates peroxidation of proteins, lipids, carbohydrates and DNA injury, introducing neuronal toxicity and also causes neuronal cell death. The biomarker of oxidative stress is lipid peroxidation (MDA). Peroxidation of lipids leads to the production of Malondialdehyde. Observations indicate that STZ increased lipid peroxidation (MDA) level and reduced the physiological anti-oxidants (GSH and SOD) level in N2A cells (Fig. 3a-c).

Another pathological marker of AD is the death of neurons associated with the cholinergic system, which is the most acceptable AD-like pathology hypothesis. The reduction in acetylcholine level triggers a decline in neurotransmission and induces propagation of dementia-linked AD-like symptoms. So AChE activity was also assessed in cells (Fig. 4). The treatment of STZ induces an increase in AChE activity, which directed the decrease in acetylcholine level, showing that STZ treatment has produced cholinergic cell death in N2A cells.

Further, to verify the outcome of STZ-induced neurotoxicity, the effect of test drugs chrysin, ACV and Anti Alzheimer's medication rivastigmine was assessed on N2A cells. Pre-treatment of test drugs showed significant protection and restored the biochemical parameters that were altered by STZ treatment. The existence of phenolic compounds in the ACV and chrysin produced protective effects due to various pharmacological activity[8,17]. The test drugs have reduced tau phosphorylation, amyloid deposition and enhanced the SOD, GSH activity compared to STZ-treated cells. They have also reduced MDA and AChE levels, thus maintaining the anti-oxidant level compared to STZ-treated cells but not up to the control level. The pre-treatment of test drugs at 2 μM concentration reported the most significant protection against cellular toxicity, tau phosphorylation and amyloid staining and also restored the anti-oxidants markers of N2A cells.

Conclusion

All the results of *in vitro* study showed that polyphenols have curative abilities against AD-like markers. ACV produced more operative results compared with chrysin and rivastigmine. In conclusion, the outcome suggests that the neuroprotective effect of ACV is mainly associated with the modification of the cholinergic neuronal system and the modulation of oxidative stress. ACV has protected N2A cells from neurotoxicity and reduced the formation of NFTs and amyloid aggregation. Therefore, our observations at the cellular level support the claims of prior animals studies that ACV may have curative potential toward AD. For future research, we would recommend further studies at molecular levels to uncover intricate protective mechanisms.

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Conflict of interest

None

Author(s) contribution

All authors had full access to all examination results. ST has planned and performed all the experiments and prepared the draft copy of the manuscript. PMM conceptualized the study design, supervised the findings of this work, and reviewed the manuscript for final submission.

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