



Synergistic effect of folic acid and galantamine against experimentally induced oxidative stress in IMR 32 cells

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Galantamine is an active constituent obtained from *Galanthus nivalis* L., a traditional herb known for its pharmacological properties, particularly nootropic effect. Folic acid is a dietary supplement that enhances neuronal activity. Effect of galantamine and folic acid on human neuronal cells is well known. In the present study, we explored the protective effect of galantamine and folic acid, both independently as well as in combination, over antioxidant defence system and nootropic effects on human neuroblastoma cells IMR-32. The treatment galantamine, folic acid and their combination was given for 24 h and cytotoxicity study was carried out by trypan blue dye exclusion assay. Apoptosis and necrosis were observed using Propidium iodide (PI) and Hoechst double staining method. Biochemical assays viz. total protein, protein carbonyl, lipid peroxidation and glutathione were analyzed along with super oxide dismutase and catalase. Result of cytotoxicity showed dose dependent increase in percent viability and significant decrease was observed in apoptosis and necrosis. Moreover, exposure to Galantamine, Folic acid and their combination significantly decreased lipid peroxidation and protein carbonyl formation along with the enhancement in antioxidant defence mechanism. Findings of these dose reliant toxicity study of Galantamine, Folic acid and their combination suggest that these has higher potency when given together and shows synergistic effect. They also causes repair of human neuronal cells IMR-32 cells enhancing the cell viability and consumption of Galantamine and Folic acid together will help in prevention of CNS disorders and neurodegeneration.

Keywords: *Galanthus nivalis*, Nootropic, Neuroprotection, Snowdrop

Neurodegenerative diseases are one of the major health challenges especially in elderly population. It includes numerous diseases categorized by progressive loss of

neurons. Various genetic and environmental factors have been found to modulate the risk for neurodegeneration¹. Neurodegeneration brings various challenges in the day to day life of an individual leading to difficulty in the survivals of the individual several nootropic agents have shown their effect on neurodegeneration. Nootropic agents, also known as smart drugs are highly acknowledged supplements that boost the cognitive performance. Nootropics increase the mental ability like learning, memory, attention, etc.².

Galantamine hydrobromide is a tertiary alkaloid which belongs to the *Amaryllidaceae* family². It is isolated from many species including *Leucojum* species, *Narcissus* species and *Galanthus* species. It is an important therapeutic option in various diseases³, including nerve pain and poliomyelitis⁴. It is a clinically approved drug for the treatment of Alzheimer disease which acts as a CNS-AChE inhibitor and allosteric potentiating ligand of the neuronal cholinergic nicotinic receptors^{6,7}. With the route of improvement, it has emerged as a massive beneficial preference in different neurological degeneration. Galantamine has been a clinically accepted drug for the remedy of Alzheimer's sickness. The medicine half way is going approximately as an AChE inhibitor that is an expected cholinergic nicotinic receptors⁸.

Folic acid is a dietary supplement that possesses fundamental roles in CNS and related functions in all age groups⁹. It is an inexpensive and multifunctional component that has shown its benefits in prevention of CNS disorders¹⁰. It also enhances the cellular neuronal differentiation by enabling biomechanical and biochemical pathway¹¹. Folic acid has also shown promising effects in functional neuronal recovery¹². It also possesses neuroprotective function as it inhibits the neuronal apoptosis via microRNAs¹³. In the current study, we tried to evaluate the neuroprotective effect of galantamine and folic acid on H₂O₂ and glutathione induced cytotoxicity in IMR-32 cells, which mimic cerebral cortex for better biological correlation.

Materials and Methods

Chemicals

All chemicals were procured from Hi Media, Mumbai, India and Sigma-Aldrich, USA.

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Cell culture and Treatment

IMR-32 cell line was obtained from National Center for Cell Science (NCCS), Pune, India. The Cell line was maintained in Minimal Essential Media (MEM) supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids and 10% Fetal bovine serum (HiMedia, Mumbai, India) in CO₂ incubator at 37°C with 5% CO₂.

Cytotoxicity testing by Trypan Blue dye exclusion assay

In our previous study, results of MTT assay showed that LC₅₀ of galantamine and folic acid after 24 h of treatment to IMR-32 cells was 7 mM¹⁴. Based on this data, three different doses (2.5, 5 and 10 mM) of galantamine and folic acid, respectively were selected to evaluate the cytotoxic effects on human neuroblastoma cell line IMR-32 by trypan blue dye exclusion assay¹⁵.

Apoptosis and Necrosis observation by Propidium iodide (PI) and Hoechst 33342 Double staining

Cultures of IMR-32 cells were set up on coverslips for different experimental groups (Table 1) and after 24 h of galantamine and folic acid treatment (2.5, 5 and 10 mM) apoptosis and necrosis were observed using PI- 1 µL (10 mg/mL) and Hoechst 33342- 1 µL (10 mg/mL). Cells were examined under fluorescence microscope¹⁶ and the percentage of apoptotic and necrotic cells were calculated^{17,18}.

Percentage of Apoptotic Cells = $\frac{LA+DA}{LN+LA+DN+DA} \times 100$ and Percentage of Necrotic Cells = $\frac{DN}{LN+LA+DN+DA} \times 100$ where, LN= live cells with normal nuclei (PI/Hoechst 33342: blue chromatin with organized structure); LA= live cells with apoptotic nuclei (PI/ Hoechst 33342: bright blue chromatin that is highly condensed or fragmented); DN= dead cells with normal nuclei (PI/ Hoechst 33342: pink chromatin with organized structure); and DA= dead cells with apoptotic nuclei (PI/ Hoechst 33342: bright pink chromatin that is highly condensed or fragmented).

Oxidative stress indices

IMR-32 cells were seeded in 12-Well plates (10⁵ cells/Well) and cultured for 24 h. After 24 h of galantamine, folic acid and galantamine + folic acid treatment respectively, these cells were used to make cell lysate. The cells were trypsinized and treated with lysis buffer (pH 7.5) containing 1% Triton X-100, 130 mM NaCl, 10 mM Tris-HCl and 10 mM NaH₂PO₄¹⁹. The mixture was incubated for 30 min at 4°C. The supernatant was used for biochemical assays like total protein (TP)²⁰, protein carbonyl (PC)²¹, lipid

peroxidation (LPO)²² and total glutathione (GSH)²³ along with the activity of superoxide dismutase (SOD)²⁴ and catalase (CAT)²⁵.

Results

Cell viability study

The effect of galantamine on cell viability was significant trypan blue dye exclusion assay. The exposure of IMR-32 cells at lower doses galantamine was somewhat similar to the control group i.e. 89.9% cell viability. At increasing dose (5 mM), it was exactly similar to the control group i.e., 90.6%, live cells. When compared to the higher dose of 10 mM galantamine, the cell mortality was decreased up to 5%. (Fig. 1) whereas, in the case of folic acid, the results were remarkable. The exposure to IMR-32 cells at lower doses folic acid near to the control group i.e. 90.3% cell viability. When the dose was increased i.e. 5 mM, it was unerringly comparable to the control group i.e., 94.6%, live cells. When compared to the higher dose of 10 mM folic acid, the cell mortality was decreased up to 5.7%. (Fig. 1)

While studying the synergistic effect of galantamine and folic acid, the results were impressive. The exposure of IMR-32 cells at lower doses of galantamine and folic acid showed 90.5% cell viability. When the dose was increased to 5 mM it increased to 91% of live cells. At higher dose of 10 mM, a significant decrease in cell mortality was observed up to 7%. Thus, significant synergism was observed in the IMR32 cell line by galantamine and folic acid.

Apoptosis and Necrosis Observation

Galantamine and Folic acid treatment with lower dose i.e. 2.5 mM showed non-variant decrease in apoptosis, whereas at higher doses i.e. 5 and 10 mM,

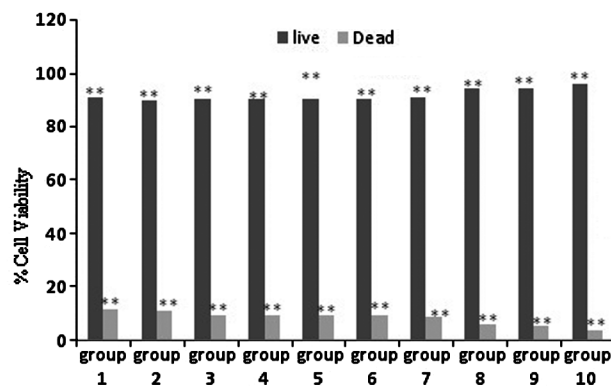


Fig. 1—Effect on Live and Dead cells after trypan blue dye exclusion assay in control (Gr. 1); and Galantamine (Gr. 2, 5, 8); Folic acid (Gr. 3, 6, 9); and Galantamine+Folic acid (Gr. 4, 7, 10) treated groups.

respectively showed potential decrease ($P < 0.001$) in apoptosis in association to the control group. Whereas the effect of galantamine and folic acid with all the three doses (2.5, 5 and 10 mM) showed effective decrease ($P < 0.001$) in necrosis when compared to control group. In the study of synergism of galantamine + folic acid at lower dose i.e. 2.5 mM, there was non-remarkable decrease in apoptosis while at higher doses i.e. 5 and 10 mM, respectively the decrease was significant ($P < 0.001$) in apoptosis when compared to the control group. In the case of effect of galantamine + folic acid on necrosis the, the decrease was noteworthy with all the three doses ($P < 0.001$) in comparison to the control group (Table 1).

Oxidative stress analysis

Galantamine and Folic acid treatment showed an increase in total protein significantly at mid and higher doses ($P < 0.05$ and $P < 0.01$, respectively) whereas non-significant alteration was observed at low dose treatment as compared to control (Fig. 2A). Protein carbonyl can be formed inside the cell due to the breakage of protein backbone by generation of ROS or direct oxidation of amino acids and it showed no significant change at low dose of galantamine and folic acid treatment. At higher dose of galantamine and folic acid treatment the results were somewhere close to normal ($P < 0.001$) as there was no breakage of protein (Fig. 2B). In order to determine the level of malondialdehyde, the level of lipid peroxidation (LPO) was measured and it showed no change at low doses (2.5 mM) of galantamine and folic acid treatment. On the other hand the mid and high doses of galantamine and folic acid treatment (Group 8 and 9) showed promising decrease ($P < 0.001$) in LPO as compared to control (Fig. 2C). Glutathione is a major defence molecule against ROS. The level of GSH was observed

to be increasing significantly ($P < 0.05$, $P < 0.01$ and $P < 0.001$), respectively for 2.5, 5 and 10 mM doses of galantamine and folic acid treatment in dose dependent manner as compared to control. These parameters showed an excellent linear dose response relationship in cultured IMR-32 cells after 24 h of galantamine and folic acid exposure (Fig. 2D). Superoxide dismutase is an enzyme that catalysis the dismutation of O_2^- into oxygen and H_2O_2 whereas, catalase converts H_2O_2 to non-toxic water molecule. The SOD activity was non prominent in any groups ($P < 0.001$) in comparison to control (Fig. 2E). The CAT activity was noticed to moderately increase ($P < 0.01$) for the group of cells treated with 10 mM galantamine and folic acid treatment (Group 10) (Fig. 2F). While studying the synergism of galantamine + folic acid value of total protein was high in mid and higher doses ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 2A). Protein carbonyl was low as there was no breakage of protein (Fig. 2B). The lipid peroxidation (LPO) level was apparently negligible at any dose (Fig. 2C). The GSH level showed significant increase (Fig. 2D). The SOD activity in galantamine + folic acid was absolutely non variant in any groups ($P < 0.001$) in comparison to control (Fig. 6). The activity of CAT was increased notably ($P < 0.01$) for the group of cells treated with 10 mM galantamine and folic acid treatment (Group 10) (Fig. 2F).

Discussion

Neurodegenerative disorders are varied group of diseases affecting the nervous system, including the brain, spinal cord and peripheral nerves that have different etiologies²⁶. Due to the prevalence, morbidity and mortality of the neurodegenerative diseases, they represent significant medical, social and financial burden to the society²⁷. Galantamine is a phytoconstituent that acts as a neuroprotective agent. Galantamine is a natural alkaloid having antioxidant properties. Galantamine is an inhibitor of acetylcholinesterase and has allosteric actions on nicotinic receptors. It is a scavenger of reactive oxygen species and exerts neuroprotection mainly by inhibition of the oxidative damage. It also alters the level of glutamate level in the brain that is involved in destruction of the neurons²⁸. Folic acid is the most important dietary supplement that is well studied for protective effect on neuronal function²⁹. It has also shown beneficial role in CNS and related activities³⁰. It is multifunctional component that has shown its benefits in prevention of CNS disorders³¹. It enhances

Table 1 — Percentage of apoptosis and necrosis by Propidium iodide and Hoechst double staining method

Groups	Apoptosis	Necrosis
Control (Gr. 1)	6.3±0.66	13.1±1.2
Galantamine low dose (2.5 mM) (Gr. 2)	6.6±0.37	13.36±1.5
Folic Acid low dose (2.5 mM) (Gr. 3)	6.0±1.3	11.2±1.6
Galantamine+ Folic acid low dose (2.5+2.5 mM) (Gr. 4)	5.8±1.4	12.1±2.2
Galantamine mid dose (5 mM) (Gr. 5)	1.9±0.17	12.02±2.1
Folic acid mid dose (5 mM) (Gr. 6)	5.7±0.2	11.9±1.1
Galantamine+Folic acid mid dose (5+5 mM) (Gr. 7)	4.9±1.1	10.7±1.3
Galantamine high dose (10 mM) (Gr. 8)	4.1±1.0	8.7±1.3
Folic acid high dose (10 mM) (Gr. 9)	4.0±1.9	8.8±1.2
Galantamine+Folic acid high dose (10+10 mM) (Gr. 10)	3.9±0.9	7.9±1.0

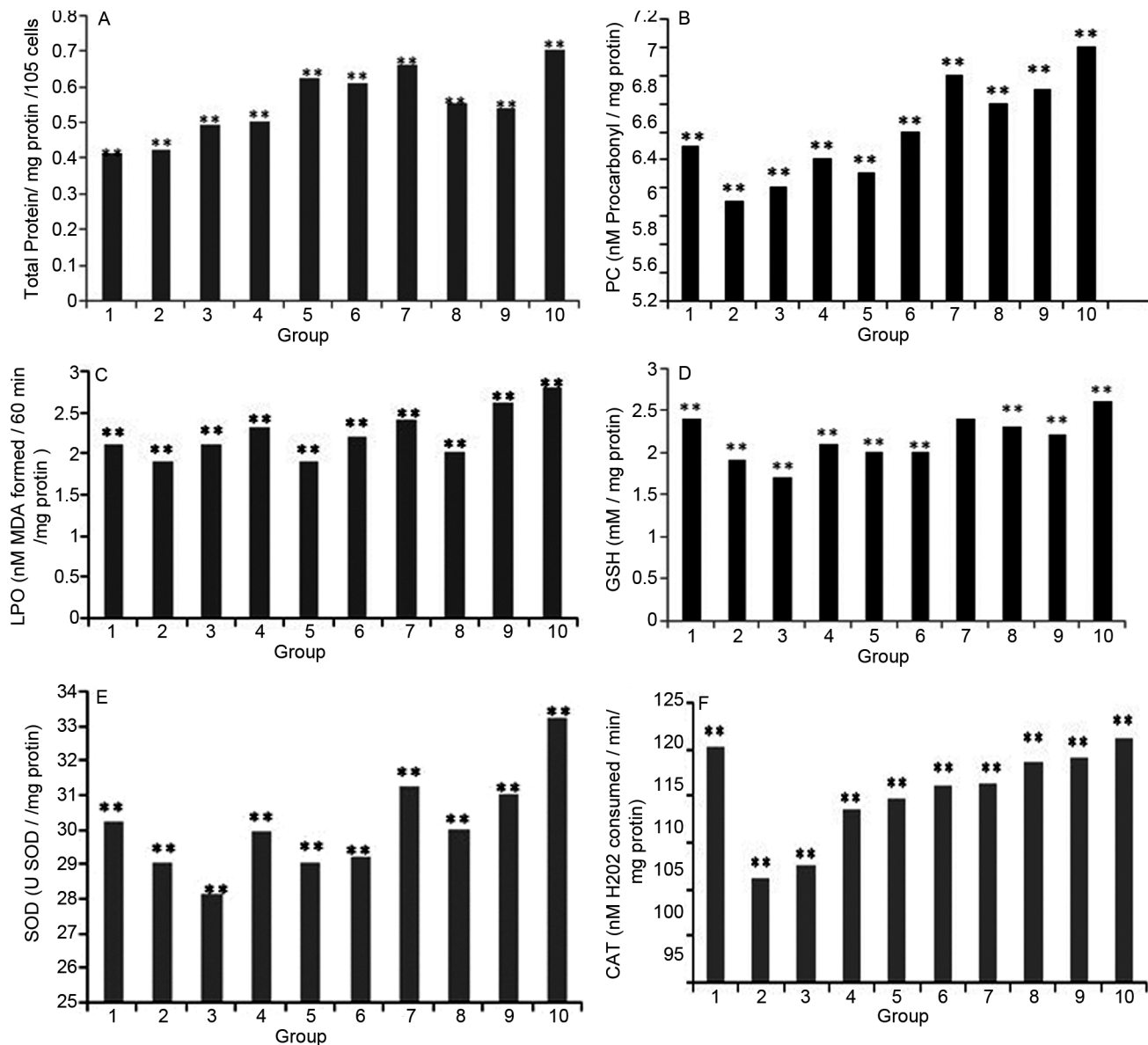


Fig. 2 — Effect on the Levels of (A) Total protein; (B) Protein carbonyl; (C) Lipid peroxidation; (D) GSH; (E) SOD; and (F) CAT in control (Gr. 1); and Galantamine (Gr. 2, 5, 8); Folic acid (Gr. 3, 6, 9); and Galantamine+Folic acid (Gr. 4, 7, 10) treated groups at low, mild and high dose with @2, 5, 10 mM, respectively [Values are expressed as mean± SEM ($P < 0.01$) and ($P < 0.001$)]

the cellular neuronal differentiation by enabling biomechanical and biochemical pathway³².

Folic acid has also shown its notable effects in functional neuronal recovery³³. It also possesses neuroprotective function as it inhibits the neuronal apoptosis via micro RNAs³⁴. Folic acid is an essential source of the single carbon group used in DNA methylation and plays a pivotal role in the development, function, regeneration, and repair of the central nervous system (CNS)³⁵. In this study, the level of glutamate was found to be elevated in cultured human neuroblastoma cell line IMR-32 studied using

MTT assay. The cell viability was negligible and the cell mortality was decreased up to 5% in case of galantamine treated group whereas it was 5.6% in folic acid treated group but even more promising result was seen in case of galantamine + folic acid treated group i.e. up to 7% which exlaimed the synergistic neuroprotective and neuroregenerative effect. The ratio of live and dead cells clearly showed that treatment of galantamine, folic acid and galantamine + folic acid treated group increased cell viability decreasing the rate of mortality. Moreover, propidium iodide and Hoechst 33342 double staining results revealed that

galantamine, folic acid and galantamine + folic acid treated group reduces apoptosis as well as necrosis in dose dependent manner. Excitotoxicity exerted by glutamate is also responsible for increase in the cytosolic Ca^{2+} level, which is due to either influx from the extracellular space or release from the intracellular stores³⁶. In this condition the survival of a cell depends largely on functioning of the mitochondria³⁷.

As mitochondria not only satisfy the cellular energy demands but also involved in ROS generation, which in turn are suspected to cause cell death if they get out of control³⁸. Increased ROS level causes damage to cell in several ways and oxidation of macromolecules like lipid, proteins and DNA. Increase in lipid peroxidation level due to ROS production results in loss of function and integrity of neuronal cell membranes, which in turn results in increase in non-specific permeability to ions, leading to disruption of membrane structure and cell functions³⁹. The malondialdehyde, a marker of lipid peroxidation was decreased significantly in galantamine + folic acid treated group, as well as in galantamine and folic acid treated groups. Oxidation of proteins and amino acids induced by ROS generation also resulted in increase of protein carbonyl level and these could be determined by carbonyl groups (aldehydes and ketones) that are produced on protein side chains when they are oxidized⁴⁰. In this study, a decrease in protein carbonyl level with corresponding increase in total protein confirms the notable rise in neuronal protein content. Antioxidant defense system consist mainly glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) as they are protagonist in our body⁴¹. The combination of Galantamine and folic acid protects the alteration of the antioxidant defense system and reduces oxidative stress. GSH protects the cells against free radical peroxides and other toxic compounds⁴².

In current study, dose dependent increment of GSH level was found after galantamine and folic acid treatment. Superoxide dismutase defends the cell against free radical injury by converting O_2^- radical to hydrogen peroxide (H_2O_2) and prevents the formation of OH^- radicals through O_2^- driven Fenton reactions⁴³. The H_2O_2 formed by SOD is removed by catalase. Hence, if the activity of CAT is not adequate to degrade H_2O_2 into H_2O and O_2 , than more H_2O_2 is converted into toxic hydroxyl radicals and finally responsible for cellular damage^{44,45}. Here, galantamine with folic acid was found to increase GSH levels as well as adhere the SOD and CAT activities, ultimately leading to the inhibition of neuronal cell damage.

Conclusion

The above results have demonstrated significance of both galantamine and folic acid treatments in neuroprotection. Galantamine and folic acid combination has shown more promising results in neuroprotection and as well as oxidative stress as indicated by cell viability and decreased apoptotic and necrotic behaviour of the cells. Apparent changes in the levels of total protein, protein carbonyl, lipid peroxidation, GSH, SOD and CAT in the treated groups have further shown synergistic effect of increased neuroprotection and decreased damage to the cells caused by oxidative stress. The neuro-protective effect of galantamine and folic acid on H_2O_2 and glutathione induced cytotoxicity in IMR-32 cells have also been shown.

Conflict of interest

Authors declare no competing interests.

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