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Modulation of MAPK Phosphorylation and Cell Viability in the T47D Breast Cancer Cell Line by treatment of Deprenyl

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Abstract: Deprenyl, a monoamine oxidase inhibitor can disrupt neuroendocrine-immune interaction as it protects some cell types against oxidative stress and has been used as a drug for the treatment of Parkinson and Alzheimer disease. Present study was to check the effect of deprenyl on T47D breast cancer cell line and Mitogen Activated Protein Kinase (MAPK) pathway. Results were obtained by giving different doses of deprenyl to Estrogen (ER) positive T47D cell line with different concentration (10^{-3}, 10^{-6} and 10^{-9} M) at different time interval (24, 48 and 72 h) and during this it has been noticed that 10^{-6} M concentration of the drug showed more viability at 72 h incubation as compared to 10^{-3} M and 10^{-9} M concentration of the same drug that showed toxic effects and necrosis, respectively. Western blotting was done with the 50 μg protein, isolated from the same cell line to check the effect of deprenyl on MAPK signaling pathway to establish at which particular concentration, the pathway is getting activated. From the results of western blotting, it was observed that deprenyl activated MAPK at 10^{-3} M concentration and activation level increased with more incubation period at same concentration of the drug that showed cellular proliferation in T47D cell line at this particular concentration of the deprenyl.

Key words: T47D, stress, deprenyl, MAPK

INTRODUCTION

American cancer society reports show that 7.6 million people died from cancer (a deadly disease in which cells will show uncontrolled growth, metastasis and invasion) worldwide during 2007. Breast cancer is one of the dangerous diseases and is the second common type of cancer worldwide. Breast cancer can be observed in aged women and mainly in developed countries. There are several risk factors for breast cancer which includes stress (Thaker *et al*., 2006), age, gender, diet and lifestyle (Simonian and Coyle, 1996), ethnicity, family history, inherited susceptibility gene and endocrine factors.

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Among all, stress is one of the major cause for breast cancer. Scientists have suggested that the effects of stress on the immune system may in turn affect the growth of some tumor (De Vasconcelos *et al*., 2001; Schapira, 1999). Age is also a main cause of breast cancer (Kitani *et al*., 2002; Shelley *et al*., 2005). Earlier puberty and social isolation were the other major factors that lead to breast cancer (McClintock *et al*., 2005).

Recent advances in our understanding of the complex signaling pathways involved in immune system regulation have accelerated development of drug-based (agonist and antagonist) strategies for cancer treatment and prevention (Salih and Fentiman, 2001). Breast cancer in women, is a complex process involving the neuroendocrine system and the immune system. There is evidence linking stress, concomitant behavioral response patterns and resultant neurotransmitter changes.

Stress is implicated in immune modulation. Bidirectional talk between immune system and endocrine system occur so that the normal functioning of the body is to be maintained in cancer. Stress leads to reduced state of immune activity. Chronic stress has been shown to suppress different facets of immune function such as antigen presentation, T-cell proliferation, humoral and cell-mediated immunity, mainly through the release of catecholamine and/or glucocorticoid hormone (Salih and Fentiman, 2001).

Deprenyl has been used as a drug for the treatment of Parkinson and Alzheimer disease. A lot of research has been done on rats and they did not showed any body weight change as compare to control. Deprenyl is an irreversible inhibitor of MAO-B (Monoamine Oxidase), which has been used as an adjunct to reduce the oxidation of dopamine. Deprenyl will influence the activation of growth factors synthesis (Kitani *et al*., 2002; Keri *et al*., 2003) resulting in regeneration of neurons and neuroprotection of cell bodies. It is a monoamine oxidase inhibitor that leads to improvement of immunological responses (ThyagaRajan *et al*., 2000). It has been observed that L-deprenyl protects these normal human uroepithelial explants via the expression of Bcl-2 protein (Seymour *et al*., 2003).

Studies have been conducted in animal models to understand the pathogenesis of disease and therapeutic effect of various drugs. Human breast cancer cell lines have also been used to understand the cellular mechanism of the disease. The MCF-7 is the most commonly used breast cancer cell line that was derived in Michigan Cancer Foundation, 1973. Apart from that there are many other cell lines that are in use viz., MDA-MB 231, T47D and Hs578T (Kabbinejadian *et al*., 2008). Among them T47D cell line is an Estrogen Receptor (ER) positive cell line and grows faster. It can grow in the absence of estrogen and frequently up regulate signaling molecules such as Raf- intracellular pathways (Hoshino, 1999; Cowley *et al*., 1994).

Mitogen Activated Protein Kinase (MAPK) pathway links extracellular signals with cellular response and influences to control essential functions such as growth proliferation, differentiation and apoptosis (Lim *et al*., 2006). There are few factors that will induce intracellular pathways like mitogen, trophin, stress and cytokines. In that mitogen and trophin will bind to tyrosine kinase and induce MAPK (Rozengurt, 2007). In higher animals, RKIP-1 has been reported to inhibit serine proteases such as thrombin and chymotrypsin, despite the fact that it has no apparent homology to any known family of serine protease inhibitors (Sartor *et al*., 1997). The MAPK can activate nuclear transcription factor (NF-kB) and can influence diverse cellular functions like cell proliferation, differentiation and apoptosis.

Since studies on breast cancer have been limited because of the complication in their pathways and other factors, therefore, present study investigated the effect of deprenyl on viability of T47D cell line by MTT assay and the effect of deprenyl on MAPK pathway.

MATERIALS AND METHODS

Chemicals

RPMI-1640 media was procured from Himedia. L-Glutamine, Penicillin, Streptomycin, Gentamycin, HEPES, Sodium Pyruvate, Bovine Insulin, Fetal Bovine Serum Deprenyl, Phosphate Buffer Saline, Trypsin- EDTA and MTT were obtained from Sigma in the month of April, 2008. Western Blotting Kit was purchased from Genie, Bangalore. Primary antibody was procured from Invitrogen. T47D human breast cancer cell lines were purchased from ATCC (American Type Culture Collection) in the second week of May, 2008.

Cell Culture

In the 3rd week of July, 2008, cell culturing was performed with slight modifications; T47D cells were maintained in RPMI-1640 in 75 cm^2 tissue culture flasks and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were fed on alternate days and passaged upon reaching 70-80% confluency (usually between 2-3 days). Viability of cells were checked by using Trypan blue method. Trypan blue is a dye that is taken up by dead cells and viable cells appear clear under the microscope.

Cryopreserving Cells

Numbers of cells were calculated by haemocytometer and $2x10^6$ cells per cryovials were stored. Each cryovial contained 1 ml freezing medium (90% FBS and 10% DMSO).

Formula to count the cells by haemocytometer = Average No. of cells×Dilution factor×10^4

MTT Assay

MTT assay was performed to check the percentage cell viability which was done in the month of September, 2008. Three different 96 well plates (24, 48 and 72 h incubation period) were seeded with 5000-7,000 cells/well (90 μL) in all the wells. 100 μL of medium was added in control. Each plate was incubated with three different concentrations (10^{-3}, 10^{-6} and 10^{-9} M) of deprenyl. No drug was added in the control. After particular incubation periods, 20 μL MTT solution was added in all the wells and plates were placed in CO_2 incubator for 3-4 h to get the purple precipitate. After getting purple precipitate, 100 μL of DMSO with 10% SDS was added to all wells, including controls and then were wrapped with aluminum foil. Plates were incubated for 2 h in dark at 37°C and the readings were taken at 450 nm and calculations were done by using SPSS 10.0 software for one way ANOVA with $p = 0.5$ and graph were plotted on excel sheet with statistical error.

Western Blotting

Western Blotting was performed to check the effect of deprenyl on MAPK signaling pathway, done in the month of December, 2008 and January, 2009. Specific antibodies were purchased from invitrogen and all other chemicals were purchased from Genie. Three different plates were prepared like MTT assay with different concentration of deprenyl (10^{-3}, 10^{-6} and 10^{-9} M) for three different incubation periods (24, 48 and 72 h, respectively). After particular incubation periods protein were lysed with lysis buffer (20 mM Tris, 100 mM NaCl, 1 mM EDTA and 0.5% Triton X 100) and protein sample was quantified by Bradford method.

Western blotting was done to check the activation of Mitogen Activated Protein Kinase (MAPK) pathway by different concentrations of deprenyl at different incubation periods.

The 30-50 µg protein sample was taken to perform the SDS Page then bands were obtained that were transferred on nitrocellulose membrane and western blotting analysis was done by antibodies specific for the phosphorylation of MAPK. Cells alone were used for the control to identify the effects of different concentrations of deprenyl on T47D human breast cancer cell lines.

RESULTS

Cell Culturing

The 1.68 million cells mL^{-1} were counted by haemocytometer and fed with 20 mL RPMI-1640 complete growth media in 75 cm^2 tissue culture flask to start the cell culturing. Cells reached almost 70-80% confluency in 36 h (Fig. 1).

MTT Assay

From MTT assay it was identified that deprenyl at 10^{-3} M concentration was toxic and cells died as compared to the control but it was noticed that as concentration was decreased to 10^{-6} M there was an increased in cell viability which decreased again slightly at 10^{-9} M. While, MTT assay was done at different time incubation it was noticed that deprenyl showed good percentage cell viability at same range and that was increased with increase in incubation period.

Results of 24, 48 and 72 h Incubation Period

Readings were taken by ELISA reader at 450 nm and one way ANOVA test was performed by SPSS 10.0 software (Table 1).

Table 1: ANOVA readings of cells treated with deprenyl for 24, 48 and 72 h incubation period

	Incubation period (h)		
Concentration of drug	24	48	72
Control	1.16±0.028	1.18±0.04	1.29±0.06
10^{-1} M	1.11±0.032	0.92±0.33	1.17±0.10
10^{-6} M	1.22±0.053	1.40±0.23	1.43±0.07
10^{-9} M	1.2±0.059	1.18±0.09	1.33±0.04

Data is expressed as Mean±SD

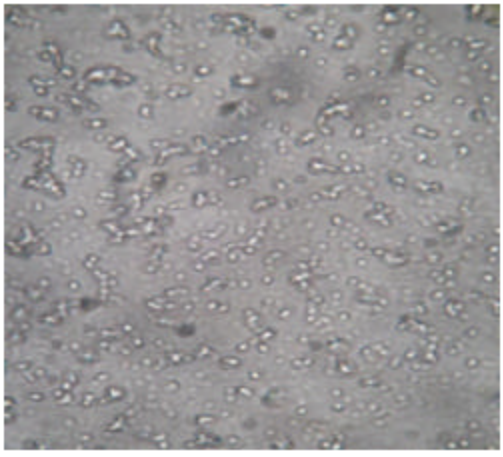

Fig. 1: Cells after 36 h of inoculation shows 70-80% confluency

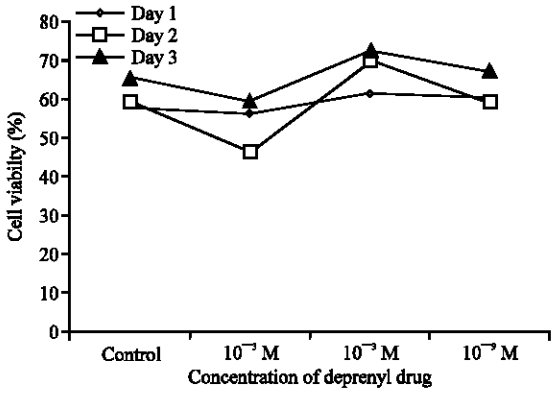

Fig. 2: Comparative graph of percent cell viability and different Deprenyl concentration over varying incubation period

24 h Incubation

Plates treated with 10^{-6} M concentration of deprenyl showed more viability as compared to cells treated with 10^{-3} and 10^{-9} M concentration of deprenyl. 10^{-3} M concentration showed least viability.

48 h Incubation

From Table 1 it can be concluded that 10^{-6} M deprenyl showed highest viability followed by 10^{-9} and 10^{-3} M concentration of deprenyl. Results indicate that overall viability was increased after 48 h of incubation as compared to 24 h of incubation.

72 h Incubation

From the table it was proved that 10^{-3} M concentration of deprenyl was toxic for cells because at this concentration cells died as compare to the control. 10^{-6} M concentration was noticed as best suited for cell viability as compare to other concentrations of the deprenyl.

Significance of Different Concentrations and Incubation Periods

From Fig. 2 it can be clearly observed that 10^{-6} M deprenyl treated cells showed maximum viability at all incubation period. Cells treated with 10^{-3} M concentration of drug showed maximum viability in 72 h incubation period. It can be seen that there was an increase in the percent viability of cells for 10^{-6} M concentration. From the graph it can be concluded that for all incubation period the viability of 10^{-9} M cells decreases as compared to 10^{-6} M deprenyl treated drug and for 10^{-3} M the viability is less. It was also observed that percent viability of cells is maximum for all the treated concentration of drug in 72 h incubation.

Western Blotting

In Fig. 3, cells were treated with deprenyl for 24 h incubation period for western blotting analysis to check the effect on MAPK. From the bands it was noticed that MAPK pathway was phosphorylated at 10^{-3} M concentration. From the bands (Fig. 4, 5) it was noticed that MAPK pathway was phosphorylated at 10^{-3} M concentration and it was increased with 48 and 72 h incubation period, respectively. The 10^{-6} and 10^{-9} M concentrations did not show appreciate level of activated MAPK. These results showed that MAPK was

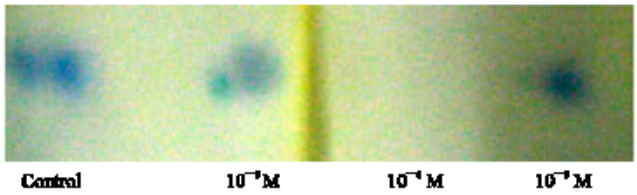

Fig. 3: Western Blotting analysis after 24 h of incubation at varying concentration of drug

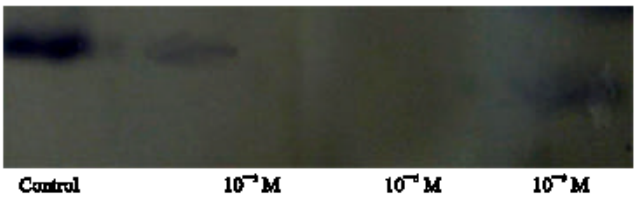

Fig. 4: Western Blotting analysis after 48 h of incubation at varying concentration of drug

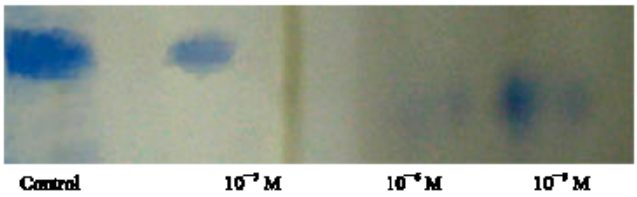

Fig. 5: Western Blotting analysis after 72 h of incubation at varying concentration of drug

phosphorylated at 10^{-3} M concentration of deprenyl and its activation was increased with the increase in incubation period (48 and 72 h, respectively).

DISCUSSION

Stress and age are the major causes for breast cancer. Researchers have revealed that treatment with deprenyl leads to enhance central and peripheral catecholaminergic activity and a readjustment of immunological response (Harman, 1956; Yu, 1996). Deprenyl was mainly used in aging and age-associated disorders (Seniuk *et al*., 1994). Deprenyl is a monoamine oxidase inhibitor and at proper dose it protects the cells from oxidative stress and apoptosis (Knoll, 1988; Runden *et al*., 1998). As it has been noticed that deprenyl did not show any body weight change as compared to control when given to rats and researchers have shown that deprenyl enhances the cells survivability and its effect increases mainly during ageing as well as age related disorder (Kitani *et al*., 1999). Because of this quality, deprenyl has been proved as a drug without any cheese effect, if given to a longer period and studies on Parkinson's and Alzheimer's patient have shown that it is a suitable drug for treating these diseases (Knoll, 1988). Studies on rodents suggested that the activity of deprenyl significantly increases with age (Kitani *et al*., 1994). Results proved that deprenyl at 10^{-4} M concentration showed more cell viability with increase in incubation periods (48 and 72 h, respectively). Our results were in line with the previous findings that long incubation of deprenyl with proper dose increases the lifespan.

Western blotting was done to check the activation of Mitogen Activated Protein Kinase pathway by deprenyl. SDS Page was started with 50 μg protein to run the gel. Cells alone were run as a control to compare the effect of deprenyl on MAPK pathway. From the results it was observed that at 10^{-3} M concentration deprenyl showed dark band as compared to other selective concentrations that showed the activation of MAPK pathway at this particular concentration of deprenyl. The activation of MAPK showed that at 10^{-3} M concentration deprenyl must have cross the cellular membrane to elicit its stimulatory effect. As research suggested that specific cell lines were responsible for the activation of specific intracellular signaling pathways (Heiser *et al*., 2009). Results suggested that 10^{-3} M concentration of deprenyl was best suited for the activation of MAPK that increased with longer incubation period (48 and 72 h, respectively).

CONCLUSIONS

The aim of present investigation was to check the effects of deprenyl on T47D human breast cancer cell lines and MAPK signaling pathway. To study this MTT and Western blotting assays have been done to check the viability of T47D human breast cancer cell lines in the presence of different doses of deprenyl and to check the activation of MAPK by different doses of deprenyl respectively. Both the assays have been performed at three different incubation periods (24, 48 and 72 h, respectively).

MTT results showed that 10^{-3} M concentration of deprenyl was toxic for cells and because at this concentration cells died as compare to the control. Deprenyl at 10^{-6} M concentration was noticed as best suited for cell viability as compare to other concentrations of the deprenyl. It was observed that at 10^{-6} M concentration cell viability increased with increase in incubation periods (48 and 72 h, espectively).

Activation of MAPK pathway was analyzed by western blotting. Activation of MAPK pathway was observed at 10^{-3} M concentration of deprenyl and as incubation period was increased, there was an increase in activation level. These findings suggested that at selective dose (10^{-6} M), deprenyl increased the cell viability which increased further with longer incubation period and the selective dose of deprenyl (10^{-3} M) activated MAPK signaling pathway that showed cellular proliferation in T47D human breast cancer cell lines.

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