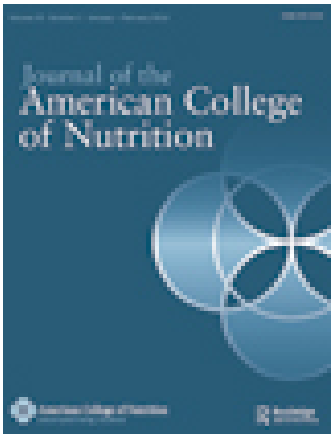

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Original Research

Potentiation of the Effect of Paclitaxel and Carboplatin by Antioxidant Mixture on Human Lung Cancer H520 Cells

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Key words: antioxidant mixture, chemotherapy, lung cancer, India, apoptosis

Objective: Antioxidants have been shown to enhance the effect of certain chemotherapeutic agents on tumor cells in culture. However, this effect differs depending upon the type of tumor and the drugs. In this study, the objective was to see whether pretreatment with antioxidant mixture could enhance the cytotoxic and apoptotic effect of commonly used chemotherapeutic agents, paclitaxel and carboplatin for the treatment of NSCLC.

Methods: Human lung squamous cell carcinoma cell line, H520, was treated with antioxidant mixture (vitamin C, vitamin E and beta carotene), paclitaxel and carboplatin, individually and in combination of different doses in different sequences. Growth inhibition and induction of apoptosis was studied by morphological changes, MTT assay and flow-cytometric analysis.

Results: The antioxidant mixture by itself led to 15% apoptosis in H520 cells. Paclitaxel treatment 24 hours prior to carboplatin caused 54% apoptosis, more than that produced by simultaneous treatment with both agents (40%). A statistically significant improvement in the degree of apoptosis, induced by paclitaxel and carboplatin combination, was seen when the cells were pretreated with antioxidant mixture immediately before paclitaxel exposure (70%) or 24 hours before paclitaxel exposure (89%).

Conclusion: The data suggests that the apoptotic effects of paclitaxel and carboplatin are enhanced by pretreatment with the antioxidant mixture. Thus, the most promising sequence of these agents, which emerged in this study, was pretreatment with antioxidant mixture for 24 hours followed by paclitaxel treatment for 24 hours followed by carboplatin exposure for 24 hours.

INTRODUCTION

Non small cell lung cancer (NSCLC) is a frequent malignant disease throughout the world including in India [1,2]. Oxidants present in smoke and pollution have been increasingly implicated in the development of lung cancer. Most of the patients present in advanced stage to an oncologist, beyond curative surgical resection [3]. Standard chemotherapy and radiotherapy produces less than desirable results [4]. Also normal cells are adversely affected by chemotherapy, leading to unavoidable, toxic side effects; many patients are unable to complete the chemotherapy. The response rate (25% to 30%) and overall survival (six to nine months) continues to be poor despite availability of newer chemotherapeutic agents including paclitaxel, gemcitabine and vinorelbine [4]. There is, thus, a need to develop newer treatment strategies to manage NSCLC, which are cost effective, spare normal cells, yet effectively destroy the cancer cells.

Though controversial, there is a possibility that in certain doses and combination, antioxidants may prevent the development of cancer [5,6]. *In vitro* studies indicate that vitamin C, vitamin E, retinoids and beta carotene individually or in combination may even induce cell differentiation and/or growth inhibition of human tumorigenic parotid acinar cells and human melanoma cells [5]. They have also been shown to enhance the growth-inhibitory effects of chemotherapeutic agents and x-radiation on human melanoma cells and murine neuroblastoma cells in culture [5]. A few animal studies support the observation that high dose of antioxidants inhibit the growth of tumors,

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such as oral carcinoma in hamsters and transplanted breast adenocarcinoma in rats [7,8].

These effects of antioxidants appear to be related to alterations in the expression of several oncogenes and growth regulatory genes [5]. Vitamins have also been found to induce apoptosis in the cancer cells while protecting the normal cells against the apoptosis induced by various agents [9–15]. The extent and type of effect depend on the dose and type of vitamins and the type of the tumor [5].

The present study was undertaken to evaluate the role of these vitamins in conjunction with the standard chemotherapeutic agents for NSCLC, i.e. paclitaxel, an antimicrotubule agent, and carboplatin, which forms DNA adducts. An MTT (3-4, 5-dimethylthiazol-2, 5-diphenyl tetrazolium bromide) assay was done to find out the optimum dose of these agents that produces the maximum killing effect in NSCLC cells. Flow-cytometric analysis was carried out to study whether apoptosis induced by carboplatin and paclitaxel in combination could be enhanced by pretreatment with an antioxidant mixture in human lung cancer cells.

MATERIALS AND METHODS

Human lung squamous cell carcinoma cell line, H520, was chosen as this is the most common type of lung cancer in India. We chose a mixture of three antioxidant vitamins/provitamins because it had been reported earlier that the mixture is more effective than individual vitamins and a mixture of at least three antioxidant vitamins/provitamins is essential for this effect [5].

Cell Culture

H520 cell line was procured form the National Center for Cell Sciences (Pune, India). The cells were grown in RPMI 1640 media supplemented with 10% fetal bovine serum and maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

Treatment of Cells

Dose range of paclitaxel was chosen on the basis of previous studies and previously standardized doses in which it was shown that a dose of 0.05 to 0.1 μmol was capable of inducing significant biological effects *in vitro* without being toxic [16]. Similarly, the effective and non-toxic dose of carboplatin reported *in vitro* is 0.5–1.5 μg/mL [17]. Two dose levels of antioxidant mixture were used in our study (Table 1). Dose level 1 was based upon earlier data [5] while dose level 2 was used to see whether doubling the dose enhances the efficacy of the mixture. The doses chosen for the antioxidants are commensurate with normal physiological serum levels.

MTT Assay for Cell Survival

Cells were seeded in 96 well plates at a density of 1×10^4 cells per well. The following day, the media was changed and some cells were left untreated (control). Cells in other wells were treated with paclitaxel or carboplatin at various doses (Table 2) within the effective dose range described above and antioxidant mixture at both dose levels (Table 1) to find out the optimum doses of these agents for further experiments. After treatment, viable cells were measured using MTT assay according to the procedures described earlier [18] and analyzed in an ELISA plate reader at 570 nm with a reference wavelength of 655 nm. Triplicate wells were taken for each treatment, and all the experiments were repeated three times.

Table 1. Dose of antioxidant mixture

Agents	Dose levels	
	Level 1 (μg/mL)	Level 2 (μg/mL)
Vitamin C	100	200
Vitamin E	10	20
Beta Carotene	10	20

Flow-Cytometric Analysis of Cell Cycle and Apoptosis

After the establishment of the optimum doses (Table 2), cells were exposed to a paclitaxel, carboplatin and antioxidant mixture in various combinations and sequences in duplicate and each experiment was repeated three times (Table 3). The cells were washed twice with phosphate buffer saline (PBS), and cell pellets were fixed in 70% alcohol and incubated for one hour at 4°C. The cells were then washed twice with 2 mL PBS each time and then resuspended in 100 μL PBS with 100 μL RNase solution (1 in 10 dilution of stock solution containing 10 mM Tris, 15 mM NaCl and 10 μg/mL RNase) and 200 μL of propidium iodide solution (100 μg/mL in PBS) and incubated at 4°C for 30 minutes. The cells were then analyzed by doing flow-cytometry with COULTER EPICS-XL flow-cytometer using 488 nm for excitation and red fluoroscence measured at 600 nm. The data was analysed using Win MDI 2.8 software.

Table 2. Effect of paclitaxel, carboplatin and antioxidant mixture on squamous cell lung cancer (H520) cells

Agents	Doses	% Cytotoxicity (Mean ± SE*)
Control	—	4 ± 1
Paclitaxel	0.05	87 ± 3
(μmol)	0.075	84 ± 2
	1.1	83 ± 3
Carboplatin	0.5	22 ± 1
(μg/mL)	1.0	22 ± 3
	1.5	22 ± 2
Vitamins	Dose level 1	15 ± 3
	Dose level 2	16 ± 3

* SE = standard error. Results are of three separate experiments, each performed in triplicate.

Table 3. Flow-cytometric analysis of the effect of combination of the agents (paclitaxel, carboplatin and antioxidant mixture) on apoptosis in H520 cells

Serial No.	Treatment of Cells				Apoptosis (% cells) (Mean ± SE*) (Day 5)
	Day 1	Day 2	Day 3	Day 4	
1	Cells plated	—	—	—	20.6 ± 1.2
2	Cells plated	Paclitaxel + Carboplatin	—	—	40.3 ± 3.1
3	Cells plated	Paclitaxel	Carboplatin	—	54.3 ± 2.2
4	Cells plated	Vitamins + Paclitaxel	Carboplatin	—	70.11 ± 3.7
5	Cells plated	Vitamins	Paclitaxel	Carboplatin	89.15 ± 4.3

* SE = Standard error. Results are of three separate experiments, each performed in duplicate.

Cells were plated on Day 1 and flow-cytometry was performed on Day 5. Control = Serial no. 1. Doses: Paclitaxel: 0.05 μmol/mL, Carboplatin: 0.5 μg/mL, Vitamin C: 100 μg/mL, Vitamin E: 10 μg/mL, beta-carotene: 10 μg/mL.

RESULTS

MTT Assay

Antioxidant Mixture Induces Cytotoxicity. Treatment of H520 cells for 24 hours with the combination of vitamin C, vitamin E and β-carotene induced cytotoxicity as observed by MTT assay. Similar levels of cytotoxicity were obtained at both high dose and low dose of this mixture, i.e., 16% and 15%, respectively (Table 2). As there was no advantage of doubling the dose, it was decided to use the lower dose level for further experiments.

Paclitaxel Induces A High Level of Cytotoxicity. By itself, paclitaxel exposure for 24 hours caused a similar degree of cytotoxicity, i.e., 83% to 87%, over the entire dose range of 0.05–0.1 μmol. We therefore chose to do further experiments with the lowest dose of 0.05 μmol of paclitaxel (Table 2).

Induction of Cytotoxicity by Carboplatin. Treatment of H520 cells for 24 hours with various doses of carboplatin, ranging from 0.5 to 1.5 μg/mL, caused a similar degree of cytotoxicity (about 22%). So we decided to do further studies with the minimum dose of carboplatin, i.e., 0.5 μg/mL (Table 2).

Flow-Cytometric Analysis

Effects of a Paclitaxel and Carboplatin Combination. Combination of minimum effective doses of paclitaxel (0.05 μmol) and carboplatin (0.5 μg/mL) were used in different sequences to analyze the most effective sequence of treatment with these drugs (Table 3), and the results were analyzed by flow-cytometry. Apoptosis was found to be significantly higher ($p = 0.047$) when paclitaxel was added 24 hours prior to carboplatin (54.3%, Fig. 1c), as compared to when both were added simultaneously (40.3%, Fig. 1b) (Table 3).

Modification of the Effect of Paclitaxel/Carboplatin Combination by Antioxidant Mixture. Pretreatment with antioxidant mixture consistently enhanced the degree of apoptosis induced by paclitaxel/carboplatin combination on flow-cytometric analysis (Table 3, Fig. 1d and 1e). Apoptosis was enhanced significantly ($p = 0.022$) from 54.30% (Fig. 1c) to 70.01% (Fig. 1d) when the mixture was added just before paclitaxel (and followed 24 hours later by carboplatin). Maximum apoptosis (89.15%) was, however, observed (Fig. 1e) when the mixture was added 24 hours before the treatment with paclitaxel/carboplatin combination ($p = 0.014$).

DISCUSSION

Paclitaxel and carboplatin have been identified as active agents in the management of NSCLC. The effective *in vitro* dose of paclitaxel has been reported to be 0.05 to 0.1 μmol [16] and that of carboplatin is 0.5–1.5 μg/mL [17]. The results of MTT assay in our study revealed that the total cell death in H520 cells remains uniform (approximately 88% with paclitaxel and approximately 22% with carboplatin) over the entire effective dose range of these agents.

Due to their different mechanisms of action and non-overlapping toxicity profile, paclitaxel and carboplatin have frequently been combined in NSCLC management. Pre-clinical and *in vitro* studies of paclitaxel and the platinum group of compounds have shown that the result of their combination depends largely upon the sequence and schedule in which these drugs are given, and maximum effect is observed when the carboplatin treatment is given 24 hours after the paclitaxel exposure [16].

It is well established that most chemotherapeutic agents and radiotherapy eliminate cancer cells by inducing apoptosis. We, therefore, did flow-cytometry to find out the level of apoptosis induced by paclitaxel and carboplatin combination. The results confirmed the observation of earlier studies that treatment with carboplatin 24 hours after paclitaxel is a better option as compared to simultaneous treatment with both the agents. A statistically significant ($p = 0.047$) level of apoptosis was observed when carboplatin was given 24 hours after paclitaxel treatment (54.30%), as compared to when carboplatin and paclitaxel were given simultaneously (40.30%) (Fig. 1, Table 3).

Various studies have investigated the role of antioxidant vitamins in the induction and regulation of apoptosis in cancer cells [9–15]. Individual vitamins/provitamins can induce direct or indirect apoptosis in cancer cells; for example, retinoic acid

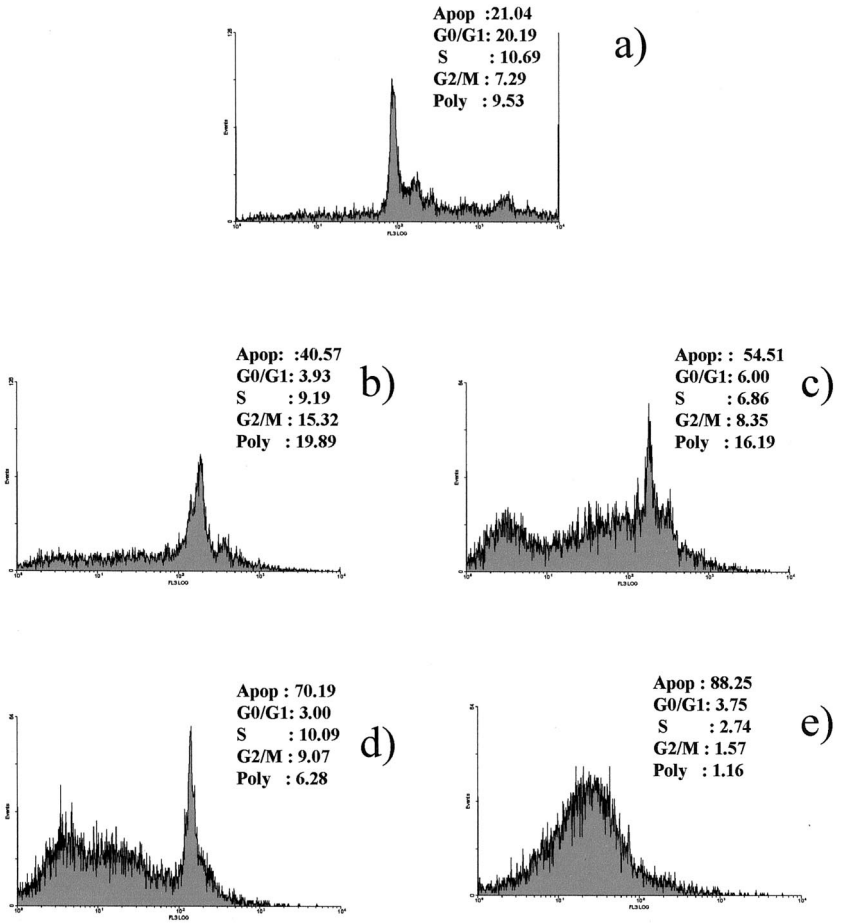

Fig. 1. Flow-cytometric analysis of the effect of combination of the agents (paclitaxel, carboplatin and antioxidant mixture) on apoptosis in H520 cells. Cells were treated as described in Table 3. Shown are the histograms of a representative experiment. **a)** control, **b)** paclitaxel and carboplatin simultaneously, **c)** paclitaxel followed 24 hours later by carboplatin, **d)** antioxidant mixture and paclitaxel simultaneously followed 24 hours later by carboplatin, **e)** antioxidant mixture followed 24 hours later by paclitaxel and 48 hours later by carboplatin.

and α-tocopherol succinate (α-TS) causes apoptosis in human B lymphoma cells in culture [11]. The agents, however, can also stimulate antiapoptotic events in certain cancer cells, and these effects depend upon the dose, type and form of the agent and the type of tumor cell [9]. A mixture of antioxidant vitamins/provitamins is more effective than individual vitamin/provitamin in the induction of apoptosis, and there is no evidence that such a mixture ever stimulates anti-apoptotic events in cancer cells [9]. Antioxidant vitamins/provitamins may enhance the effect of direct acting apoptotic agents (x-rays, chemotherapeutic agents and hyperthermia) or indirect acting apoptotic agents (adenosine 3,5 cyclic monophosphate, butyric acid and interferon) and, in combination with these agents, produce a greater extent of apoptosis in cancer cells than these agents alone [19].

In contrast to vitamin/provitamin induced apoptosis in cancer cells, normal cells never undergo apoptosis after treatment with these agents (excluding retinoids) [9,20]. On the contrary, they protect normal cells against apoptosis induced by certain group of chemicals [9, 12–15, 20]. For example, α-TS protected spinal cord organotypic cultures against etanidazole induced apoptosis [12], epidermoid cell culture against ultravoilet B induced apoptosis [13], and neuronal cultures against oxidative stress induced apoptosis [14]. In addition to vitamin E, retinoic acid also protects against activation induced T cell apoptosis and thus plays a role in the regulation of T cell development [15]. The reasons for this differential effect of vitamins are not known, and the genetic regulation of apoptosis in cancer cells has not been adequately defined.

To ascertain the effect of antioxidant vitamins/provitamins on H520 cells in this study, the cells were exposed to two dose levels (Table 1) of these agents, and their effect was studied using MTT assay. The antioxidants induced approximately 15% cytotoxicity in these cells and doubling the dose of the mixture did not improve the efficacy (Table 2). Further experiments were, therefore, carried out using the lower dose level only.

To evaluate the appropriate sequence, H520 cells were exposed to the antioxidant mixture immediately or 24 hours

prior to paclitaxel/carboplatin treatment (Table 3, Fig. 1). Pretreatment with an antioxidant mixture enhanced the level of apoptosis induced by paclitaxel/carboplatin combination consistently (Table 3, Fig. 1). When the mixture was added immediately before paclitaxel, the level of apoptosis rose to 70.11% (Fig. 1d), as compared to maximum apoptosis (54.3%) (Fig. 1c) observed with a paclitaxel/carboplatin combination ($p = 0.022$). However, when the mixture was added 24 hours prior to paclitaxel, the level of apoptosis significantly rose to 89.15% (Fig. 1e) ($p = 0.014$).

These observations suggest that antioxidant vitamins/provitamins with paclitaxel and carboplatin therapy may have utility in the treatment of lung cancer. Pretreatment with antioxidant mixture followed by paclitaxel and carboplatin exhibited greater than additive effect indicating a potential synergistic combination for NSCLC. This combination thus may provide more efficacious treatment of non-small cell lung cancer.

The results of our study show that the antioxidant vitamins/provitamins induce cytotoxicity by themselves in H520 cells. These agents also enhance the level of apoptosis induced by the combination of paclitaxel and carboplatin. Thus it seems that the response rate to this chemotherapy regimen in the management of NSCLC might be improved with the addition of an antioxidant mixture. A higher level of apoptosis has been found to be associated with longer survival in NSCLC [21]. The mechanism of action of these agents in the induction of apoptosis is not very clear. Further studies need to be carried out to investigate this aspect.

Another rationale for using antioxidants in combination with chemotherapeutic agents involves the possible reduction of toxicity of several chemotherapeutic agents on normal cells by these agents [22–35]. The antioxidant mixture is more effective than the individual components in protecting normal cells against radiation damage [35]. This particular aspect, however, was not evaluated in the present study.

CONCLUSION

An antioxidant vitamins/provitamin mixture appears to be potentially useful in the management of NSCLC. The mixture enhances the apoptosis induced by standard chemotherapeutic agents employed for the management of NSCLC, i.e., paclitaxel and carboplatin. Since the mechanism of the action of the components of this antioxidant mixture is not entirely clear, this needs to be studied; also, there is a need to initiate clinical trials to determine the *in vivo* effect of these antioxidants in conjunction with chemotherapy in the management of human cancers.

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