Polish Journal of Environmental Studies Vol. 14, No. 3 (2005), 341-346

Original Research

Amifostine Antioxidant Effect on Serum of Rats Treated with Cyclophosphodamide

A. Stankiewicz, E. Skrzydlewska*

Department of Analytical Chemistry, Medical Academy of Białystok, P.O. Box 14, 15-230 Białystok, Poland

Received: 4 March, 2004
Accepted: 1 December, 2004

Abstract

Amifostine is one of the cytoprotective drugs used during anticancer therapy. Amifostine as a thiol compound possesses antioxidant properties and protects only healthy cells against damage, mainly by scavenging reactivity oxygen species, competing with oxygen to prevent oxygen radical interactions with DNA, and promoting cell repair through hydrogen donation to reactive oxygen species. The aim of the present study was to evaluate antioxidative ability of amifostine in blood serum of rats exposed to cyclophosphamide during two weeks after drug administration. We show that amifostine only to a small degree prevents disorganisation of antioxidant systems of blood serum of rats caused by cyclophosphamide action. It is probably connected with low concentrations of amifostine active metabolites in the serum.

Keywords: blood serum, amifostine, cyclophosphamide, antioxidative ability, lipid peroxidation

Introduction

Cyclophosphamide, a bifunctional alkylating compound, is the most widely used oxazaphosphorine in the treatment of many neoplastic diseases: breast carcinoma, acute lymphoblastic leukaemia, nonHodgkin's lymphoma and a variety of bone and soft tissue sarcomas [1-3]. Cycloposphamide is inactive until it is metabolized by cytochrome P450 in the liver and to a smaller degree in the lung to yield phosphoramide mustard and acrolein, which may alkylate DNA and modify proteins [4]. During activation of cyclophosphamide by cytochrome P450, generation of reactive oxygen species such as superoxide anions was observed [5-6]. Cyclophosphamide can also be metabolized by prostaglandin H synthase and horseradish peroxidase to active metabolites, possibly by a free radical mechanism [7]. Moreover, cyclophosphamide can also be cooxidized by lipoxygenases [8]. Activity of lipoxygenases has been demonstrated in blood cells, lung, brain and spleen [8]. In consequence of cyclophosphamide metabolism, acrolein and phosphoramide mustard are generated. It has been suggested that cyclophosphamide metabolites may be degraded with the generation of reactive oxygen species. Because all cyclophosphamide metabolites, but reactive oxygen species in particular, are reactive compounds they can modify components of malignant as well as healthy cells [9]. It has been shown that cyclophosphamide induces liver and lung damage as well as cardiotoxicity and nephrotoxicity [10-11]. Therefore, cytoprotection of healthy tissues is needed. One of cytoprotective drugs used in anticancer therapy is amifostine. Amifostine [S-2-(3-aminopropylamino) ethyl phosphorothioic acid], designated WR-2721, is aminothiol that is used in clinical practice as a protective compound against chemotherapy-related cytotoxicites [4]. Amifostine is an inactive prodrug, which is activated to metabolite – WR-1065 by membrane-bound alkaline phosphatase, whose activity is highest in healthy cells of intestine, liver, heart, kidney and red blood cells [12-13]. All the mechanisms of cytoprotection of healthy cells by amifostine have not been fully elucidated, but the most significant seems to be WR-1065, which is active thiol and acts as an intracellular scavenger of reactive oxygen species [14].

*Corresponding author; e-mail: skrzydle@amb.edu.pl

Table 1. The activity of antioxidative enzymes in the blood serum of control rats (C) and rats treated with cyclophosphamide (CP), amifostine (A) and cyclophosphamide and amifostine (CP+A).

Enzyme	Time after injection (days)	Control	CP	A	CP+A
SOD U/mg protein	1	0.51 ± 0.04	0.24 ± 0.02 [a]	0.54 ± 0.05	0.30 ± 0.02 [a b c]
	5	0.52 ± 0.03	0.39 ± 0.01 [a]	0.53 ± 0.03	0.46 ± 0.04 [a b c]
	14	0.49 ± 0.05	0.36 ± 0.03 [a]	0.48 ± 0.02	0.41 ± 0.01 [a b c]
GSH-Px U/mg protein	1	0.35 ± 0.02	0.24 ± 0.01 [a]	0.42 ± 0.03 [a]	0.38 ± 0.03 [b c]
	5	0.37 ± 0.01	0.21 ± 0.02 [a]	0.40 ± 0.05	0.42 ± 0.01 [a b]
	14	0.33 ± 0.01	0.22 ± 0.02 [a]	0.35 ± 0.02	0.39 ± 0.02 [a b c]
GSSG-R mU/mg protein	1	27 ± 3.0	19 ± 2.0 [a]	31 ± 3.0	30 ± 1.0 [b]
	5	31 ± 2.0	30 ± 4.0	42 ± 4.0 [a]	27 ± 2.0 [c]
	14	29 ± 4.0	25 ± 5.0	26 ± 2.0	28 ± 2.0

Data points represent means ± SD; n=6; [a] $p<0.05$ in comparison with control group; [b] $p<0.05$ in comparison with cyclophosphamide group; [c] $p<0.05$ in comparison with amifostine group;

In this study we analyzed the concentration or activity of various compounds involved in defence mechanisms against reactive oxygen species and results of oxidative stress expressed as lipid peroxidation products, in blood serum of rats receiving high-dose cyclophosphamide and amifostine.

Material and Methods

Male Wistar rats (approximately 230 g b.w.) fed on a standard diet were used for the experiment. The experimental protocol was approved by the ethical committee on human and animal experimentation of our medical academy.

The rats were divided into 4 groups:

A. Control group (n=18).
B. Cyclophosphamide group. The rats were treated intraperitoneally with cyclophosphamide in doses of 150 mg/kg b.w. (n=18).
C. Amifostine group. The rats received intraperitoneally amifostine at 200 mg/kg b.w. (n=18).
D. Cyclophosphamide and amifostine group. The rats were treated intraperitoneally with amifostine at 200 mg/kg b.w. and subsequently with cyclophosphamide at 150 mg/kg b.w. (n=18)

1, 5, and 14 days after drug treatments the rats were sacrificed following ether anaesthesia (six animals in each group). Blood taken from the heart was incubated at 37°C and centrifuged to obtain serum blood.

Superoxide dismutase (SOD; EC.1.15.1.1) activity was determined by the method of Misra and Fridovich [15] as modified by Sykes [16]. Glutathione peroxidase (GSH-Px; EC.1.11.1.6) activity was measured spectrophotometrically using a technique based on Paglia and Valentine [17]. Glutathione reductase (GSSG-R; EC.1.6.4.2) activity was measured by monitoring the oxidation of NADPH at 340 nm [18]. Reduced glutathione (GSH) concentration was measured using Bioxytech GSH-400 test (Bioxytech S.A., Bonneuil/Marne Cedex, France). High-performance liquid chromatography (HPLC) methods were used to determine the level of vitamin C [19] and vitamins A and E [20]. Total antioxidant status (TAS) was measured with 2,2'-azino-di-[3-ethylbenzthiazoline] sulphonate (ABTS) using Randox test.

Lipid peroxidation was assayed by measuring malondialdehyde (MDA) as a malondialdehyde-thiobarbituric acid adduct by HPLC and 4-hydroxynonenal (4-HNE) as a fluorimetric derivative, also using HPLC [21-22].

Statistics data obtained in this study are expressed as mean ± SD. The data were analyzed by use of standard statistical analyses, one-way ANOVA with Scheffe's F test for multiple comparisons to determine the significance between different groups. The values for $p<0.05$ were considered significant.

Results

Cyclophosphamide as well as amifostine have been shown to influence the activity of antioxidant enzymes in rat blood serum (Table 1). Administration of cyclophosphamide caused a significant decrease in superoxide dismutase (SOD) activity (from about 50% on day 1 to about 27% on day 14 of the experiment). After injection of amifostine the activity of SOD did not change significantly. However, simultaneous administration of cyclophosphamide and amifostine caused a decrease in examined enzyme activity throughout the experiment, but to a lower degree than after cyclophosphamide administration alone. Similarly to SOD case, cyclophosphamide also caused a significant decrease in the activity of glutathione peroxidase (GSH-Px) (on 1st and 14th day by about 33% and on 5th day by about 44%). However, amifostine administration led to an increase in the activity of this enzyme (by about 17%) only on day 1 of the experiment. Administration of cyclophosphamide and amifostine caused the activity of GSH-Px to increase in comparison with control group (significantly - by about 12% on day 5 and by about 16% on day 14 of the experiment). Injection of cyclophosphamide also caused a significant decrease (by about 30%) in the activity of glutathione reductase (GSSG-R) only on day 1. After administration of amifostine the activity of this enzyme was significantly increased (by about 27%)

Table 2. The level of nonenzymatic antioxidants in blood serum serum of control rats (C) and rats treated with cyclophosphamide (CP), amifostine (A) and cyclophosphamide and amifostine (CP+A).

Analysed parameter	Time after injection (days)	Control	CP	A	CP+A
GSH µmol/g tissue	1	2.70 ± 0.11	2.78 ± 0.13	2.97 ± 0.13 [a]	2.77 ± 0.21
	5	2.40 ± 0.21	4.63 ± 0.17 [a]	3.70 ± 0.15 [a]	3.81 ± 0.22 [a b]
	14	2.60 ± 0.12	3.87 ± 0.22 [a]	3.07 ± 0.17 [a]	3.60 ± 0.17 [a b c]
vitamin C µg/g tissue	1	1.66 ± 0.11	1.20 ± 0.12 [a]	1.95 ± 0.12 [a]	1.62 ± 0.10 [b c]
	5	1.56 ± 0.12	1.29 ± 0.13 [a]	1.89 ± 0.14 [a]	1.53 ± 0.12 [b c]
	14	1.60 ± 0.10	1.39 ± 0.10 [a]	1.90 ± 0.11 [a]	1.52 ± 0.13 [b c]
vitamin A nmol/g tissue	1	0.61 ± 0.02	0.21 ± 0.04 [a]	0.38 ± 0.03 [a]	0.47 ± 0.03 [a b c]
	5	0.62 ± 0.03	0.23 ± 0.06 [a]	1.02 ± 0.07 [a]	0.43 ± 0.04 [a b c]
	14	0.60 ± 0.01	0.25 ± 0.07 [a]	1.22 ± 0.05 [a]	0.42 ± 0.05 [a b c]
vitamin E nmol/g tissue	1	13.52 ± 1.1	11.60 ± 1.0 [a]	18.31 ± 1.2 [a]	11.11 ± 1.5 [a c]
	5	13.30 ± 1.3	10.20 ± 1.7 [a]	20.20 ± 1.5 [a]	15.35 ± 1.3 [a b c]
	14	13.40 ± 1.0	12.80 ± 1.1	19.70 ± 1.0 [a]	13.52 ± 1.0 [c]
TAS nmol/g tissue	1	1.11 ± 0.12	0.67 ± 0.03 [a]	1.23 ± 0.09	1.12 ±0.09 [b]
	5	1.01 ± 0.11	0.52 ± 0.02 [a]	1.37 ± 0.12 [a]	1.27 ± 0.08 [a b]
	14	1.07 ± 0.07	0.58 ± 0.03 [a]	1.35 ± 0.14 [a]	1.10 ± 0.11 [b c]

Data points represent means ± SD; n=6; [a] $p<0.05$ in comparison with control group; [b] $p<0.05$ in comparison with cyclophosphamide group; [c] $p<0.05$ in comparison with amifostine group;

on day 5. After injection of cyclophosphamide and amifostine, GSSG-R activity did not change in comparison with control group.

Changes in antioxidant enzyme activity observed after cyclophosphamide and/or amifostine administration were accompanied by changes in nonenzymatic antioxidant levels and all these changes differ in intensification through all the experiments (Table 2). Cyclophosphamide caused a significant increase in reduced glutathione (GSH) levels in rat blood serum (by about 48% on day 5 and by about 33% on day 14). After injection of amifostine the level of GSH was significantly increased on the 1st (by about 10%), 5th (by about 36%) and 14th (by about 15%) days. Administration of cyclophosphamide and amifostine also caused a significant increase in the level of reduced glutathione on the 5th (by about 38%) and 14th (by about 40%) days. Cyclophosphamide caused a significant decrease in the level of vitamins in blood serum of rats. Vitamin C concentration was diminished by about 28%, 18% and 14% on the 1st, 5th and 14th days, respectively. After administration of the protecting drug, the level of this antioxidant increased by about 15% on the 1st and 14th days and by about 18% on the 5th day. Injection of cyclophosphamide and amifostine did not significantly change the level of this vitamin in comparison with the control group. Administration of cyclophosphamide caused a significant decrease in vitamin A levels in the blood serum on the 1st (by about 66%), the 5th (by about 63%) and on the 14th days (by about 59%). After administration of amifostine the level of this vitamin was also decreased but only on the 1st day (by about 38%). During the next experimental days concentration of this antioxidant significantly increased (by about 40% and 50% on the 5th and 14th days, respectively). Administration of both examined drugs caused a significant decrease in the level of vitamin A (on the 1st day – by about 27% and on the 5th and 14th days - by about 30%). Cyclophosphamide, similar to the case of other vitamins, caused a significant decrease in vitamin E (by about 15% on the 1st day and by about 33% on the 5th day). However, injection of the protective drug was the reason for the significant decrease in the level of this vitamin (by about 26, 35 and 32% on the 1st, 5th and 14th days, respectively). Administration of cyclophosphamide and amifostine caused a significant decrease in the level of this nonenzymatic antioxidant (by about 18 and 14% on the 1st and 5th days, respectively) in comparison with control group. Cyclophosphamide leading to changes in nonenzymatic antioxidant levels caused, in consequence, a decrease (by about 40, 50 and 46% on the 1st, 5th and 14th days) in the level of total antioxidant status (TAS) in blood serum of rats. However, amifostine injection led to a significant increase in this parameter (by about 26% and 21% on the 5th and 14th days, respectively). Administration of both drugs did not significantly change the level of TAS.

It was proved that cyclophosphamide enhanced lipid peroxidation in rat blood serum, as a consequence of changes in antioxidant parameters (Table 3). Injection of cyclophosphamide caused a significant increase in the level of final lipid peroxidation product – malondialdehyde (MDA) on the 1st, 5th and 14th days (by about 38, 26 and 35%, respectively). However, administration of amifostine caused significant MDA increase only on the 5th day (by about 4%). Cyclophosphamide injected with amifostine changed MDA levels during the whole time of the experiment. On the 1st and 14th days significant increase in MDA levels were observed (by about 10 and 32%, respectively), while on the 5th day a significant decrease in the level of this aldehyde was shown (by about 17%). In the case of 4-hydroxynonenal (4-HNE), after cyclophosphamide injection the level of this compound was significantly increased on the 5th and 14th days (by about 30 and

Table 3. The level of lipid peroxidation products in the blood serum of control rats (C) and rats treated with cyclophosphamide (CP), amifostine (A) and cyclophosphamide and amifostine (CP+A).

Analyzed parameter	Time after injection (days)	Control	CP	A	CP+A
MDA nmol/ml	1	0.64 ± 0.01	1.02 ± 0.11 [a]	0.63 ± 0.02	0.71 ± 0.04 [a b c]
	5	0.67 ± 0.01	0.91 ± 0.07 [a]	0.78 ± 0.03 [a]	0.56 ± 0.04 [a b c]
	14	0.62 ± 0.03	0.94 ± 0.04 [a]	0.63 ± 0.04	0.91 ± 0.01 [a]
4-HNE nmol/ml	1	0.32 ± 0.01	0.33 ± 0.02	0.30 ± 0.02	0.35 ± 0.01 [c]
	5	0.29 ± 0.02	0.41 ± 0.04 [a]	0.28 ± 0.01	0.31 ± 0.03 [b c]
	14	0.31 ± 0.02	0.39 ± 0.03 [a]	0.27 ± 0.01 [a]	0.36 ± 0.02 [a c]

Data points represent means ± SD; n=6; [a] $p<0.05$ in comparison with control group; [b] $p<0.05$ in comparison with cyclophosphamide group; [c] $p<0.05$ in comparison with amifostine group;

21%, respectively). Administration of amifostine caused significant decrease in the level of 4-HNE only on the 14th day (by about 13%). Cyclophosphamide and amifostine caused an increase in the above parameter also on the 14th day of the experiment (by about 14%).

Disscusion

Anticancer therapy with cytostatic drugs has been used for many years. Nevertheless, many mechanisms resulting in damage of healthy cells have not yet been examinated. Cytostatic metabolites and reactive oxygen species generated during these drugs' metabolism are considered to participate in such mechanisms [23-24]. It is known that enhanced generation of reactive oxygen species causes changes in cell redox balance, which leads to oxidative stress, resulting in cancer and healthy cell damage [25]. It has been proved that oxidative stress leads to injury of: bone marrow, gastrointestinal tract, kidney, urinary bladder, lung, nervous system and vasculary system healthy cells [26-27]. Reactive oxygen species also are produced during cyclophosphamide metabolism in reactions catalyzed both by cytochrom P450 and by peroxidases and lipooxygenases [7]. Moreover, it was observed that Cu, Zn-SOD or α–tokoferol supplementation reduces active metabolite production during cyclophosphamide metabolism catalyzed by lipooxygenases [7]. This confirms participation of free radical mechanisms in cyclophosphamide metabolism.

The above data indicate that antioxidant defence mechanisms must take part in toxicity of cyclophosphamide [5]. Durken et al. (1995) have shown that patients receiving high-dose cyclophosphamide displayed significant reductions of antioxidant parameters in plasma [28]. In the present study many changes in rat blood serum antioxidative systems also have been observed after cyclophosphamide administration. Cyclophosphamide injection caused decrease in the activity of antixidant enzymes: SOD, GSH-Px and GSSG-R. It is possible that this decrease is a result of protein structure modification through the reactive metabolite acroleine and/or reactive oxygen species generated during cyclophosphamide metabolism as well. SOD selectively eliminates superoxide radicals in dismutation reaction in which hydrogen peroxide is generated. Reduction of SOD activity also may be due to an inhibited biosynthesis of enzyme molecules by cyclophosphamide or its metabolites and/or to the effect of hydrogen peroxide, which may directly alter its activity [6]. However, hydrogen peroxide may be removed by GSH-Px system, which suggests an important role of the GSH-glutathione peroxidase system in cyclophosphamide toxicity [29]. The decrease in the activity of glutathione peroxidase and in the level of reduced glutathione nearly stops removal of peroxides. It corresponds with enhanced levels of lipid peroxidation products observed after cyclophosphamide injection. A decrease in the activity of glutathione peroxidase as well as glutathione reductase might result from thiol group oxidation in the active center of these enzymes by oxygen-free radicals or acrolein [30]. However, in the case of reduced glutathione we observed an increase in the concentration of this parameter after injection of cyclophosphamide in rat blood serum. The transient increase in GSH content on the 5th and 14th days after cyclophosphamide treatment is believed to be an acute adaptive response to an oxidant injury related to this drug. GSH plays a very important role in the detoxification of drugs. In vitro examinations proved that the free thiol group of glutathione reacts with acrolein and conjugates are formed as a result of Mitchel addition [31]. These conjugates with acrolein also reveal toxic properties, though not as strong as unbound aldehyde alone [32]. In consequence, acrolein quickly depletes cellular glutathione levels and has the potential agent to inhibit many enzymes, which lead to further lipid peroxidation [33].

Apart from GSH, other nonenzymatic antioxidant parameters, vitamins in particular, play an important role in reducing serum oxidative stress, which efficiently scavenges superoxides, hydrogen peroxide, hypochloride, hydroxyl and peroxyl radicals [34-35]. Cyclophosphamide caused the diminition in the level of vitamins C, E and A in rat blood serum. The decrease in the level of vitamins is dangerous because additionaly to antioxidant function vitamins play a role in sparing other antioxidants [36]. Results obtained in this paper indicate that the reduction of total antioxidant status, which testifies to the possibility of modification cell components by reactive oxygen species.

Systemic approaches with selective protection of normal cells from the toxic effects of chemotherapy also have been investigated [37-39]. The most promising results to date have been obtained with the organic thiophosphate compound – amifostine. Amifostine appears to enter non-malignant cells selectively by facilitated diffusion, and potentially provides protection against oxygen radicals and electrophilic reactive compounds, such as cyclophosphamide metabolites [4]. Amifostine has been shown to reduce various forms of short-term toxicity of cyclophosphamide in animal models without reducing antitumor efficacy [40-42]. This drug can affect intracellular redox states, alter gene expresion, synthesis of other intracellular thiols and enzyme activities [43]. Amifostine directly prevents DNA damage though the physiochemical process of radical scavenger, chemical repair by hydrogen atom donation and induced hypoxia by autooxidation [44-46].

Amifostine is dephosphorylated to its active metabolite WR-1065 in red blood cells, kidneys, liver, small intestine and to a small degree in spleen and lungs by membrane-bound alkaline phosphatase, whose activity is very low in blood serum [4]. Maximum concentration of WR-1065 in serum was observed shortly after bolus injection of amifostine [47]. WR-1065 has been rapidly cleared from the plasma compartment with initial half-life of 0,18h followed by a slower second phase with a half-life of 7,3h. The short initial half-life can be explained by the fast uptake in tissues and approximately 90% of the drug is cleared from plasma within 6 minutes [48]. WR-1065 secretion in serum blood is thrice smaller than in liver and seven-fold smaller than in kidneys [42]. In consequence, protective ability of WR-1065 in blood serum is comparatively little, where significant protective antioxidant ability of amifostine were demonstrated, in contrast to tissues [49-50]. The present study has proved that amifostine causes a significant increase in concentration of GSH and vitamins A, E and C and in the activity of GSH-Px and GSSG-R in blood serum. However, amifostine only to a low degree reduces oxidative influence of cyclophosphamide on antioxidative enzymes and nonenzymatical parameters in blood serum. In consequence, the lipids are not significantly protected against oxidative stress.

In conclusion, amifostine protection in blood serum against oxidative stress caused by cyclophosphamide is limited, connected with low concentration of amifostine active metabolites in the serum.

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