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Inhibition of glycolysis and respiration of sarcoma-180 cells by cyclophosphamide

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The malignant cells in tumor are known to produce high level of lactic acid. The anticancer drug cyclophosphamide has been reported to have a highly promising anticancer activity against fibrosarcoma bearing rats. In the present investigation, the effect of the energy metabolism of sarcoma-180 (S-180) on the mode of action of cyclophosphamide, as well as the effect of cyclophosphamide on the mitochondrial and cellular respiration of S-180 cells was studied. The effects of glucose utilization, pyruvate utilization and lactate formation were studied on whole S-180 cells and S-180 cell-free homogenate. The levels of glycolytic enzymes such as hexokinase and lactate dehydrogenate (LDH) were estimated. The utilization of glucose and pyruvate was found to decrease which resulted in decreased formation of lactic acid. The mitochondrial respiration was also found to decrease significantly after treatment with cyclophosphamide treated cells. The activity of glycolytic enzymes and mitochondrial respiration were also found to decrease. In conclusion, cyclophosphamide affects both cellular and mitochondrial respiration, leading to reduction of cellular energy pool and thereby resulting in a loss of viability of S-180 cells.

Keywords: Cyclophosphamide, Sarcoma-180 cells, Malignant tumor, Hexokinase, Lactate dehydrogenase.

INTRODUCTION

The rate of glucose transport, alterations in the cellular levels and regulatory properties of key glycolytic enzymes are believed to be responsible for the abnormal metabolic properties of malignant tumors [1-3]. The significant increase in the rate of sugar uptake has been consistently observed in cells transformed by sarcoma viruses. In transformed-chick embryo cells, the rate of glycolysis is increased 2 to 4 fold even under conditions of rigorously controlled growth rate and cell densing [4, 5]. The rate of aerobic glycolysis in tumor cells might indicate that the key enzymes are less sensitive to normal feed back control. It has been reported that in a number of systems, stimulation of glycolytic enzymes leads to cell proliferation and deoxyribonucleic acid (DNA) synthesis [6-8]. The tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA) has been shown to activate glucose metabolism to provide the necessary precursors for cell proliferation in target tissues [9]. Several anticancer drugs have been reported to inhibit the aerobic glycolysis seen in various types of cancers [10, 11]. Cyclophosphamide has been reported to show significant anticancer effects both *in vitro* and *in vivo* [12]. Therefore, the objective of the present study was to investigate the effects of glycolysis and respiration in sarcoma-180 cells caused by cyclophosphamide.

EXPERIMENTAL

Sarcoma-180 cell lines were obtained from the National Facility for Animal Tissue and Cell Culture, Pune, India. Healthy male Swiss albino mice (20-25 g) were used as experimental animal models. The experimental mice were maintained in controlled environmental conditions of temperature and humidity. The experimental mice were maintained in an animal house with food and water given *ad libitum* (Gold Mohar M/s. Hindustan Lever Ltd., Mumbai, India). This research work on Swiss albino mice was sanctioned and approved by the Institutional Animal Ethics Committee, International Institute of Biotechnology and Toxicology, Chennai, India.

Sarcoma-180 cells were grown in the abdominal cavity of Swiss albino mice, $1X10^7$ cells were inoculated into the peritoneal cavity at the recipient mice. The cells were harvested between 8 to 10 days and washed with sterile saline. The cells were finally suspended in phosphate buffer saline (PBS) of pH 7.4.

After the exposure of S-180 cells to cyclophosphamide for various time intervals, the

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cell viability was determined by the trypan blue exclusion test [13].

Mitochondria from S-180 cells were prepared by the digitonin permeabilization method [14].

The respiratory mechanism for mitochondria of S-180 cells contained in a total volume of 2.2 ml, 0.75 ml of PBS, 125 mg of sucrose, 50 mg of KCl, 2 mg of KH_2PO_4, 1 mg of $MgCl_2$ and respiratory substrate (5 mg of glutamate plus 5 mg of malate) and mitochondria containing approximately 200 mg of protein of S-180 cells was studied. After an indicated period of time, ADP (0.4 mg) was added to start phosphorylating respiration [10].

2×10^8 of S-180 cells in 2.2 ml of PBS were sonicated for 40 seconds (in four 10-S bar) by keeping the cell suspension on ice. After sonication, the cells were centrifuged at 1500 g for 5 min and the cell pellet was discarded and the supernatant was used as S-180 cell-free homogenate.

S-180 cells were incubated with 5 mg of glucose (5 mg of pyruvate instead of glucose for pyruvate utilization study). After 1 h of incubation with cyclophosphamide at different concentrations at 37°C in a metabolic shaker, the reaction was terminated with 70% perchloric acid and the respective metabolites such as glucose [15], pyruvate [16] and lactate [17] were estimated. The results were expressed as micromoles of metabolite cells in 60 min.

S-180 cells (approximately 8×10^7cells) were suspended in PBS and incubated with or without cyclophosphamide in a total volume of 2 ml. After l h of incubation, the cells were washed twice with PBS and cell-free homogenate was prepared as described above, the activities of enzymes such as lactate dehydrogenase [18] and total hexokinase [19] were determined. The hexokinase was also separated into cytosolic and particulate forms and assayed. To separate hexokinase into soluble and particulate forms, S-180 cell-free homogenate was centrifuged at 50000 g for 10 min. The supernatant was used for the assay of cytosolic hexokinase. The pellet was suspended in PBS and again centrifuged as described above. The supernatant was discarded and the pellet was suspended in a minimum volume of PBS and used for the assay of particulate hexokinase.

RESULTS AND DISCUSSION

Table 1 shows the inhibitory effect of cyclophosphamide on glucose utilization and L-lactic acid formation from glucose by S-180 cells. Table 2 shows the effect of cyclophosphamide on pyruvate utilization and L-lactic acid formation by S-180 cells.

Table 3 shows the inhibitory effect of cyclophosphamide on glucose utilization and L – lactic acid formation from glucose by S-180 cell-free homogenate.

Table 4 shows the inhibitory effect of cyclophophamide on pyruvate utilization and L-lactic acid formation by S–180 cell-free homogenate.

Table 5 shows the inhibitory effect of cyclophosphamide on glycolytic enzymatic enzymes of S–180 cells after incubating white blood cells (WBC) with cyclophosphamide.

Table 1 Effect of cyclophosphamide on glucose utilization and L-lactic acid formation by S-180 cells: The results are expressed per 10^8 cells in 1 h (each value is a mean ± SD of 6 experiments)

Groups	Glucose utilized (μmol)	L-Lactic acid formed (μmol)
Control (Cyclophosphamide)	5.51± 0.46	3.85 ± 0.26
(1 mg)	4.98 ± 0.37	3.41 ± 0.22*
(2 mg)	4.75 ± 0.35*	3.10 ± 0.19#
(3 mg)	4.47 ± 0.33#	4.95 ± 0.17#
(5 mg)	4.11 ± 0.29#	4.71 ± 0.14#

*($p<0.05$), # ($p<0.001$)

Table 2 Effect of cyclophosphamide on pyruvate utilization and L-lactic acid formation by S-180 cells: The results are expressed per 10^8 cells in 1 h (each value is a mean ± SD of 6 experiments)

Groups	Pyruvate utilized (μmol)	L-Lactic acid formed (μmol)
Control (Cyclophosphamide)	4.76 ± 0.36	3.18 ± 0.21
Cy (1 mg)	4.37 ± 0.32	2.85 ± 0.18*
Cy(2 mg)	4.45 ± 0.28*	2.61 ± 0.15#
Cy(3 mg)	4.65 ± 0.24#	2.46 ± 0.13#
Cy(5 mg)	4.48 ± 0.22#	2.37 ± 0.12#

*($p<0.05$), # ($p<0.001$)

Table 3 Effect of cyclophosphamide on glucose utilization and L-lactic acid formation by S-180 cell-free homogenates (each value is a mean ± SD of 6 experiments)

Groups	Glucose utilized / mg protein / h, μmol	L-Lactic acid formed / mg ptm / h, μmol
Control	0.89 ± 0.06	0.35 ± 0.02
Cy (5 mg)	0.55 ± 0.04	0.24 ± 0.001#

 # (p<0.001)

Table 4 Effect of cyclophosphamide on pyruvate utilization and L-lactic acid formation by S-180 cell-free homogenates (each value is a mean ± SD of 6 experiments)

Groups	Pyruvate utilized / mg protein / h, μmol	L-Lactic acid formed / mg / protein / h, μmol
Control	0.75 ± 0.04	0.28 ± 0.02
Cy (5 mm)	0.45 ± 0.03#	0.20 ± 0.01#

(p<0.001)

Table 5 Effect of cyclophosphamide on glycolytic enzymatic enzymes of S-180 cells after incubating white blood cells with cyclophosphamide (each value is an average ± SD of 6 experiments)

Enzyme	Enzyme activity, units / mg		
	Control	Cyclophosphamide treated (3 mm)	Cyclophosphamide treated (5 mm)
Hexokinase	0.44 ± 0.04	0.29 ± 0.2#	0.18 ± 0.01#
Total			
Particulate	0.30 ± 0.02	0.22 ± 0.01#	0.091 ± 0.006#
Cytosolic	0.20 ± 0.01	0.12 ± 0.01	0.09 ± 0.01
LDH	1.68 ± 0.18	1.18 ± 0.13#	1.18 ± 1.12#

(p<0.001)

Figure 1 shows the percent viability of S-180 cells after incubating for various periods of time. From figure 1 it is evident that cyclophosphamide has a profound impact on the viability of S-180 cells.

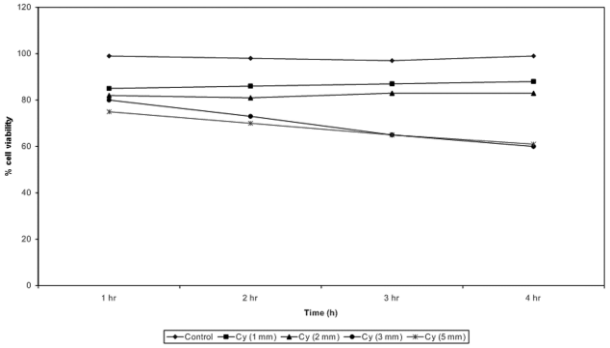

Fig. 1 Effect of cyclophosphamide on the percent viability of S-180 cells after incubating for various time intervals

Figures 2 and 3 show that cyclophosphamide inhibited both the mitochondrial and cellular respiration of S-180 cells.

Figure 4 shows the inhibitory effects of cyclophosphamide on the biosynthesis of DNA, RNA and protein in S-180 cells.

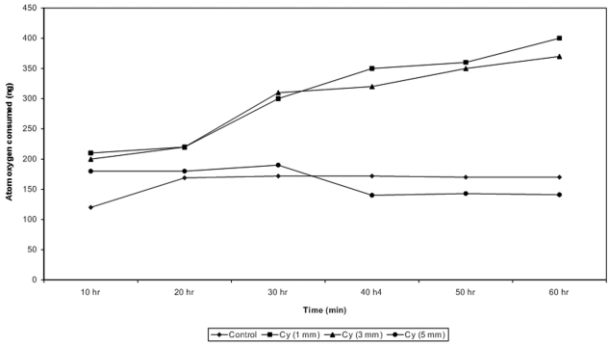

Fig. 2 Effect of cyclophosphamide on the respiration of mitochondria from control and cyclophosphamide (1, 3, 5 mm) treated S-180 cells

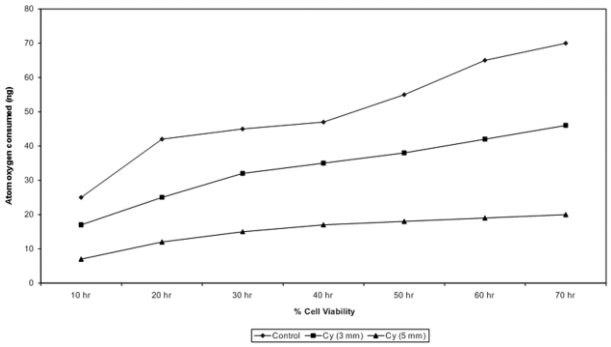

Fig. 3 Effect of cyclophosphamide on the respiration of control and cyclophosphamide (3, 5 mm) treated S-180 cells

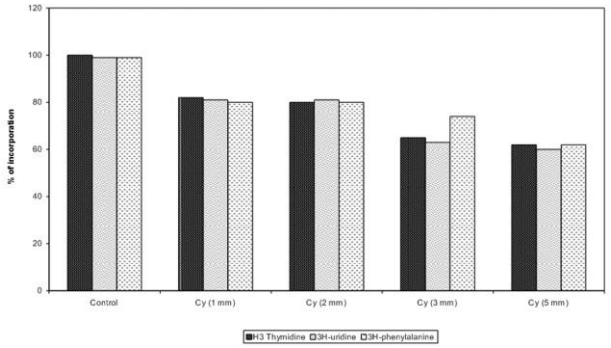

Fig. 4 Effect of cyclophosphamide on % incorporation of 3H-thymidine, 3H-uridine and 3H-phenylalanine in S-180 cells after 1 h of exposure

When incubated with cyclophosphamide, the viability of S-180 cells was found to decrease after 24 h of exposure. The decrease in the viability of S-180 cells after treatment with cyclophosphamide showed that this effect may be due to the inhibition of glycolysis and mitochondrial respiration. The enzyme hexokinase has attracted considerable attention both because it commits glucose to catabolism and it has been shown to be markedly elevated in tumors exhibiting a high glucose catabolic rate [20, 21]. The concentration of hexokinase tumor cells is higher and bound to mitochondria [22, 23]. The mitochondrial bound hexokinase has direct access to ATP generated from oxidative phosphorylation and able to phosphorylate glucose with an enhanced efficiency. Since transformed cells and tumor cells contain large amounts of bound hexokinase [24, 25] and catalyse high rates of glycolysis, the levels of total cytosolic and particulate hexokinase were determined after treatment with cyclophosphamide. The results clearly showed that the levels of both total and particulate hexokinase decreased after treatment with cyclophosphamide with no significant decrease in the cytosolic form, thereby suggesting that cyclophosphamide, even in high concentrations, inhibits glycolysis by blocking only the particulate hexokinase enzyme. Glucose–6–phosphate, a product of hexokinase catalysed reaction, is a critical precursor not only for glycolysis, which in highly glycolytic tumors, provides as much as 60% of cells ATP supply and biosynthesis of nucleic acids [26]. The decreased synthesis of DNA might have resulted in the inhibition of cell division and membrane biosynthesis, thereby restricting the cell growth.

The inhibition of glycolysis by cyclophosphamide was measured both in terms of lactate and pyruvate formation and glucose consumption. In the present study the utilization of glucose and pyruvate was found to decrease resulting in decreased formation of lactic acid. The decreased lactate levels, as observed during pyruvate utilization study, might be due to the inhibition of LDH. LDH is an important rate limiting enzyme of glycolysis. Its activity has been found to decrease significantly after treatment with cyclophosphamide. Hence, the decreased activity, as noted in the present study, is consistent with the decreased rate of glycolysis in cyclophosphamide and suggests that the LDH activity is elevated in tumors [3, 27].

The mitochondrial respiration was also found to decrease significantly after treatment with cyclophosphamide treated cells. The inactivation of these important glycolytic enzymes and mitochondrial respiration of S-180 cells by cyclophosphamide strongly suggested that this compounds act upon specific target sites, which are probably altered in tumor cells. The results of 3H-thymidine, 3H-uridine and 3H-phenylalanine clearly indicated that cyclophosphamide, apart from inhibiting glycolysis and respiration, is also able to inhibit macromolecular biosynthesis of DNA in S-180 cells. Previously this drug was shown to inhibit DNA biosynthesis in the fibrosarcoma cell line [14]. The inhibitory effects of anticancer drugs on macromolecular biosynthesis are also confirmed with Ehrlich ascites carcinoma cell line [28-31].

In the present investigation it was found that the tumor cells lost their ability to respire within minutes after addition of cyclophosphamide. Cyclophosphamide may interfere with tumor cell glycolysis and could induce cell death. As a consequence, the respiration of the malignant cells is inhibited. Thus it is more likely that the cytotoxic effects of cyclophosphamide may be probably mediated through interference with the oxidative process and energy metabolism of the tumor cell. It follows from these observations that cyclophosphamide independently and simultaneously affects both glycolysis and mitochondrial respiration, leading to reduction of the cellular energy pool, thereby reducing the viability of the S-180 cells.

CONCLUSION

The results of the present study showed that cyclophosphamide has great impact on both cellular and mitochondrial respiration of S-180 cells. These impacts cause reduction in cellular energy pool which ultimately results in the loss of viability of S-180 cells. Finally, these results indicated the strong inhibitory effects of cyclophosphamide on glycolysis and respiration of S-180 cells.

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ИНХИБИРАНЕ НА ГЛИКОЛИЗАТА И ДИШАНЕТО НА КЛЕТКИ НА САРКОМА-180 ЧРЕЗ ЦИКЛОФОСФАМИД

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(Резюме)

Известно е, че злокачествените туморни клетки проявяват висока гликолизна активност, водеща до високо производство на млечна киселина. Съобщава се, че противораковото лекарство циклофосфамид има многообещаващо противораково действие срещу фибросаркома при опитни плъхове. В настоящата работа е изследван ефектът на енергиен метаболизъм при клетки саркома-180 (S-180) при въздействието на циклофосфамид върху митохондриалното и клетъчното дишане на клетките S-180. Ефектът на използване на глюкоза, пирувати и образуването на лактати е изследван върху цели клетки S-180 cells и тяхно безклетъчни хомогеати. Оценено е нивото на гликолитичнте ензими като хексокиназа и лактат-дехидрогеназа (LDH). В настоящата работа се забелязва намаляване на утилизацията на глюкоза и пируват, което води до образуване на млечна киселина. Освен това е намерен, че митохондриалното дишане намалява значително след третиране на клетките с циклофосфамид циклофосфамид. Активността на гликолитичните ензими и митоондриалното дишане. В заключение, циклофосфамидът влияе върху клетъчното и митохондриалното дишане, водещи до намаляване клетъчния енергиен резервоар и с това до загубата на жизненост на клетките S-180.