Histopathological evaluation of tumor necrosis and volume after cyanogenic chemotherapy[1]

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ABSTRACT

PURPOSE: To determine the percentage of tumoral necrosis and volume after cyanogenic chemotherapy.

METHODS: Histopathological findings of 20 Swiss mice inoculated subcutaneously in the left abdominal wall with 0.05 ml of cell suspension containing 2.5 x 10^5 viable cells of the Ehrlich tumor were evaluated. The tumor response to cyanogenic chemotherapy was determined using a system that comprises two inhibition factors of tumor growth by calculating the percentage of necrosis in the tumor tissue and calculation of tumor volume in treated animals relative to that in control animals. The importance of this system has been validated by the correlation between tumor inhibition in the groups treated with the respective percentages of necrosis.

RESULTS: While the control group presented an average of 13.48 ± 14.71% necrosis and average tumor volume of 16.18 ± 10.94, the treated group had an average of 42.02 ± 11.58 and 6.8 ± 3.57, respectively. The tumor inhibition was significantly associated with treatment (p=0.0189). The analysis of necrosis percentage showed a significant prognostic importance (p=0.0001).

CONCLUSION: It is concluded that the effect of cyanogenic chemotherapy showed strong inhibitory action of tumor growth, as well as an increase in its area of necrosis.

Key words: Neoplasms, Chemotherapy, Antineoplastic Agents, Sulfur, Thiosulfate Sulfurtransferase, Hydrogen Cyanide, Oxidative Phosphorylation.

Introduction

Rhodanese (thiosulfate sulfotransferase) is a sulfotransferase involved in the cyanide detoxification and oxidation processes. The rhodanese carries sulfane sulfur covalently bonded to another atom of sulfur from certain sulfur donor (as thiosulfate, polysulfides and persulfides) to various acceptors, including cyanide[1,2].

The neoplastic cells have a total lack of activity of cystathionase (CST), while the activity of cysteine aminotransferase, 3-mercaptopyruvate sulfotransferase (MPST) and rhodanese is only residual. Such behavior undermines the biosynthesis and transport of sulfur compounds from these sulfane cells[3,4]. Toohey[3] suggests that the uncontrolled proliferation of cancer cells is the result of sulfur sulfane disability and hyperactivity of these enzymes, which would be inhibited in normal cells by this active form of sulfur[3]. The rhodanese also presents low activity in livers of mice bearing Ehrlich ascites tumor, due to a decrease in the levels of sulfane sulfur[5,6].

The sulfane sulfur is generated from several metabolic pathways, and often there are carrier proteins capable of stabilizing and transporting sulfur sulfane. A deficiency in the anaerobic metabolism of sulfur is related to neoplastic processes and immunodeficiency virus infection, suggesting that this may have a regulatory function in cells by modifying the -SH groups, and this high activity and short half-life characteristics confer an efficient regulator[7].

Acetone cyanohydrin (AC) through its molar equivalent of cyanide, is capable of promoting a reduction in transplanted tumors in mice and, in some cases, the total reduction of such tumors. This quite possibly occurs by a defect in the detoxification process of cyanide in tumor cells involving cell stocks of sulfur, in which they are easily intoxicated even with low doses of cyanide[8,9].

Here we present our results based on a cyanogenic chemotherapy with the use of acetone cyanohydrin as a prodrug for release of cyanide, seeking a possible failure in its metabolism in tumor cells.

Methods

The research project was approved by the Ethics Committee on the use of animals of the Federal University of Mato Grosso do Sul (UFMS), protocol number 227.

20 male mice of the Swiss strain, weighing 35±5g obtained from the animal colony of the Federal University of Mato Grosso do Sul were used. The animals were kept in experimentation for seven days prior to the procedures.

Tumor transplant

The cells were kept *in vivo* in donor mice by the intraperitoneal transplantation of $2x10^6$ Ehrlich Ascite Tumor (EAT) cells. Every ten days the formed intraperitoneal fluid was removed and transplanted in another animal.

The donor animal was euthanized with an overdose of anesthetic. After that, peritoneal lavage was performed with 3mL of phosphate buffered saline and then withdrawing by suction the same 3mL. The obtained cell suspension was centrifuged, the supernatant discarded and the pellet resuspended in 3mL of phosphate buffered saline. In suspension, the test cell viability by Trypan blue exclusion was performed. Then it was determined the number of cells in a Neubauer chamber.

All twenty animals were weighed and then sedated with 0.1 mL of ketamine/xylazine (0.5/100mg and 0.2 mg/100g respectively) and after shaving they were subcutaneously inoculated with 0.05 ml of cell suspension containing $2,5x10^5$ viable cells of the Ehrlich tumor in the left abdominal wall, this being regarded as day zero of the experiment.

Treatment program

Treatment was initiated on day 3 and repeated every 48 hours until the 13th day. Randomly, the animals were divided into 2 groups: control (CG) n = 10, treated with saline; Acetone cyanohydrin (ACG), n = 10 treated with 2,796 mg/kg AC. The doses were administered intraperitoneally. 48 hour after the last treatment, the animals were euthanized by administration of an overdose of anesthetic.

Tumor extraction

The tumor mass was collected and its measure at its longest and shortest axis was determined using a digital caliper brand ZAAS. Then the solution was maintained at 10% formaldehyde for one day and transferred to 70% alcohol. After that the samples were embedded in paraffin, cut at 5μm thick and stained with hematoxylin-eosin (HE).

Morphological evaluation in tumor inhibition

The determination of tumor inhibition was performed using the calculating tumor volume according to the following formula[10,11]:

$$TV = Lxs^2/2$$

Being:
(TV) Tumoral volume
(L) Longest axis
(s) Shortest axis.

Quantification of the percentage of tumor necrosis

The slides were stained with hematoxylin and eosin and then subjected to reading and determination of areas of necrosis; through the tumor mass, they were quantified using AutoCAD 2014 software (Free version for testing).

The images of the tumor were analyzed by microscopy in the 20X objective and captured in its entirety using a digital camera brand Nikon Coolpix model. After being scanned, the images were analyzed in AutoCAD software and areas of necrosis bounded by the electronic cursor and calculated in mm^2, also delimitating the tumor area and calculating their respective area in mm^2 mass.

For percentage of necrosis through the tumor mass, we used the following formula:

$$\% \text{ necrosis area} = (\text{necrosis area} / \text{total tumoral mass area}) * 100$$

Statistical analysis

The results were analyzed by statistical parametric type for the two independent samples with use of the t-Student test. The level of significance was 5% ($p < 0.05$).

Results

After administration of AC some adverse reactions were observed. In the ACG, the animals had intense reactions as initial agitation, lasting approximately 10 seconds, followed by convulsions culminating in animal prostration and tachycardia. These signals were seen in all treatment days.

All inoculated animals developed solid Ehrlich tumor (SET) by the seventh day after inoculation and showed varying shapes and sizes, not being possible to observe significant differences between the control group and those treated with AC. Upon removal of the tumor, they presented several different forms and sizes with whitish and smooth appearance, not yet presenting invasion of adjacent tissue.

Histologically, the tumors in the control group presented themselves as a neoplastic mass circumscribed by a pseudocapsule, where the neoplastic cells had a high degree of differentiation characterized by cellular pleomorphism and prominent nucleus. It was observed the presence of cells in the mitotic process and also moderate number of giant cells. The tumors also showed a moderate inflammatory infiltrate.

There was also, in tumors of the control group, areas of coagulative type of necrosis, where it was possible to find cells that had more eosinophilic cytoplasm and pyknotic nucleus, cells in cariorexe, i.e. with irregular chromatin distribution and accumulated in the nuclear membrane, cells with fragmented nuclei and others with dissolution of chromatin and disappearance of nuclear structure. In some tumors, we observed the presence of fibrous tissue.

At the histological aspect, tumors of ACG and CG showed to be very similar. However, it was possible to observe a significant difference between the two groups in the occurrence of coagulation necrosis and tumor volume, with an evaluation of the effect of AC on SET performed based on these values (Table 1).

TABLE 1 – Acetone Cyanohidrine effect in Solid Ehrlich Tumor in relation to tumoral necrosis and volume parameters.

	Necrosis (%)[a]	**Tumoral Volume (mm^3)[b]**
CG	13,48±14,71	16,18±10,94
ACG	42,02±11,58	6,8±3,57

Each value represents the average ± SD. (n = 10 mice per group)
[a]p = 0.0001 [b]p = 0.0189

The results of the percentage of necrosis in the group treated with AC showed a significant difference (p = 0.0001) when compared with the control group (Figure 1).

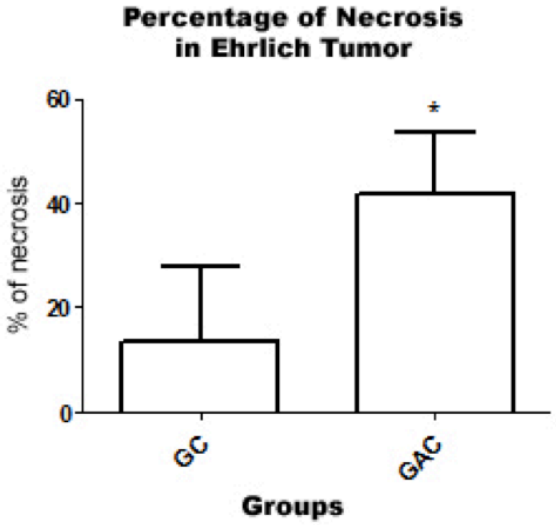

FIGURE 1 - Graph representing the average standard deviation of the percentage of necrosis in CG and ACG.
* p = 0.0001

In terms of tumor volume, results of AC-treated group also showed a significant difference (p = 0.0189) compared with the control group (Figure 2).

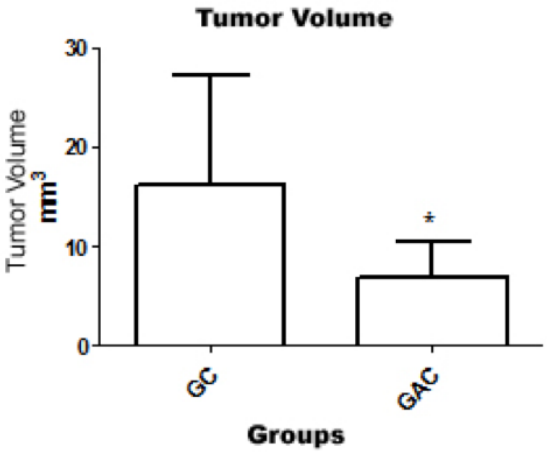

FIGURE 2 - Graph representing the average standard deviation of tumor volume in CG and ACG.
* p = 0.0001

Discussion

The ability of cyanide to penetrate the cell without the action of a receiver is due to the cyanide ion being a small molecule and its detoxification in mammals occur for the most part mediated by the enzyme rodanase[12]. Animals bearing solid tumors undergoing distance treatment with cyanide showed a clear regression of tumors compared to controls, demonstrating that cyanide was diffused through the tissues into the bloodstream[9,13]; in our experiment using SET inoculated subcutaneously in animals, cyanogenic chemotherapy released cyanide outside the tumor, abdominal cavity, but causing accentuated necrosis and tumor growth inhibition.

The cyanide poisoning can cause reactions such as seizures, tachycardia followed by prostration[14]; similar reactions were observed in this experiment after the animals were exposed to cyanogenic chemotherapy, showing once again the ability of cyanide to diffuse through the tissues, promoting its toxic action in the brain of these animals which are more susceptible tissues to the action of cyanide[1].

A feature present in neoplastic cells is the residual activity of rhodanese[15], due to a deficiency of sulfur sulfane, this fact makes the transport and biosynthesis of compounds from this group do not occur satisfactorily in such cells. This suggests that the uncontrolled proliferation of neoplastic cells is a result of deficiency of this element, and that the active form of sulfur promote inhibition of proliferation in normal cells[16]. In a previous study[9], this characteristic of tumor cells was demonstrated promoting a strong cyanide poisoning, showing a great ability to inhibit tumor growth, not being shown significant differences between the doses used.

The results obtained in this study demonstrated a direct influence of cyanide to promote an increase of necrosis and inhibition of tumor growth in animals with SET, when compared to the control group. These results suggest a differential ability of tumor cells to eliminate cyanide, probably by the activity of rhodanese be significantly reduced in these cells because of a lower content of sulfur sulfane as reported in literature[3,17,18].

The action of cyanogenic chemotherapy using AC as cyanide source in the experiment developed suggests the ability of tumor cells to irreversibly poison the action of cyanide, thereby providing a dose-and time-dependent effect[12,], as occurred in previous studies[8,9]. Cyanide toxicity occurs in two ways: ATP depletion caused by blockage of oxidative phosphorylation and production of reactive oxygen species[12].

In SET it was observed the presence of molecular changes consistent with necrosis. The presence of coagulative necrosis in SET is frequently observed, particularly in larger tumors, where necrosis is characterized by extensive cell lysis that occurs during the course of acute injury[19,20]. In the present experiment a sharper ACG necrosis was observed in relation to the CG; such lesions are consistent with exposure to cyanide, where ATP levels are suspected to fall sharply due to inhibition of oxidative phosphorylation, being this decrease below the level required for apoptosis, thus resulting in cell death by necrosis[12].

Evidence observed in the present study suggests that the selective action of the cyanide is due to a deficiency in their elimination observed in tumor cells as widely discussed in the literature.

Conclusion

The cyanogenic chemotherapy using acetone cyanohydrin as a cyanide source showed strong inhibitory action of tumor growth and an increase in its area of necrosis.

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