Effects of acetylsalicylic acid and acetic acid solutions in VX2 carcinoma cells. *In vitro* analysis[1]

Efeito da solução de ácido acetilsalicílico e de ácido acético sobre o carcinoma vx-2. Análise *in vitro*

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ABSTRACT

Purpose: To analyze, *in vitro*, the effects of acetylsalicylic acid (aspirin) and acetic acid solutions on VX2 carcinoma cells in suspension and to examine the correlation between these effects and neoplastic cell death. **Methods**: The VX2 tumor cells (10^7 cells/ml) were incubated in solutions containing differing concentrations (2.5% and 5%) of either acetylsalicylic acid or acetic acid, or in saline solution (controls). Every five minutes, cell viability was tested (using the trypan blue test) and analyzed under light microscopy. **Results**: Tumor cell viability (in %) decreased progressively and, by 30 minutes, neoplastic cell death had occurred in all solutions. **Conclusion**: Based on this experimental model and the methodology employed, we conclude that these solutions cause neoplastic cell death *in vitro*.
Key words: Carcinoma. Aspirin. Acetic Acid. In vitro.

RESUMO

Objetivo: Analisar os efeitos das soluções de ácido acetil salicílico (aspirina) e de ácido acético, in vitro, sobre células em suspensão do carcinoma VX-2, verificando-se as mesmas causam a morte das células neoplásicas. **Métodos**: Procedeu-se a incubação das células tumorais VX-2 (10^7 células/ml) com diferentes concentrações do ácido acetil salicílico (2,5% e 5%) e de ácido acético (2,5% e 5%), sendo estudada a viabilidade celular pelo teste do azul tripian a cada 5 minutos; procedeu-se à análise à microscopia ótica. **Resultados**: Observou-se que o percentual de viabilidade das células tumorais foi progressivamente diminuindo, sendo que ao final de 30 minutos todas as células neoplásicas estavam inviáveis em todas as soluções e concentrações utilizadas. **Conclusão**: Com base neste modelo experimental e com a metodologia empregada, concluiu-se que in vitro, estas soluções causam a morte (inviabilidade) das células neoplásicas.
Descritores: Carcinoma. Aspirina. Ácido Acético. In vitro.

Introduction

The human preoccupation with cancer is understandable in light of its high incidence worldwide. In Brazil, it is the second leading cause of death among the adult population[1]. Neoplasia is also the second leading cause of death in Brazilians over the age of 40 and the third leading cause of death at any age, accounting for approximately 110,000 or 16% of deaths of the deaths that occur annually[2]. The most common types of neoplasia are skin, breast, lung, stomach, uterus, colon and rectum and prostate. Together, they account for 157,000 new cases every year[2]. The main factors affecting prognosis and recurrence of neoplasia are lymph node involvement, local recurrence, striation and, especially, the presence of distant metastases. One of the most commonly affected organs is the liver. Treatment options for liver neoplasia are limited by factors such as the number of metastases and their locations. The principal modalities of treatment for hepatic metastases currently include surgical resection, arterial ligation, embolization, chemotherapy and genetic therapy. Ablation techniques involving necrotizing and cytolytic substances, lasers, radiofrequencies, microwaves, hyperthermia and cryotherapy have also been used. Surgical resection is the main treatment for neoplasia and is the only one that offers the possibility of a cure. However, only small percentages (10 – 15%) of patients are good candidates for surgical intervention[3].

Among the palliative methods, ablation stands out. In order to destroy the lesion locally, cytolytic and necrotizing substances, including alcohol, are commonly used. Other ablation methods include cryotherapy, radiofrequency, laser and microwave. Ablation methods are generally reserved for inoperable cases. In the face of these obstacles and therapeutic considerations, new treatments for hepatic metastases should be developed. Such treatments should present high rates of efficacy, low cost, low occurrence of

side effects and should be easily executed. In a study performed in 1996[4], we analyzed the effects of a solution consisting of acetic acid, glycerin, phenol and distilled water on Ehrlich ascites tumor cells *in vitro* and *in vivo* and observed that the solution caused tumor cell death *in vitro*. Intraperitoneal injection of the solution *in vivo* reduced the number of tumor cells and increased the number of inflammatory cells in suspension in the acetic acid solution[4].

In light of this observed effect, we decided to analyze the possible effects of one or more of the solution components in isolation. In the literature, there are no references to the use of acetic acid, or of its derivative, acetylsalicylic acid, in experimental tumors. Considering this fact and the results previously obtained, we decided to evaluate the *in vitro* effects of acetylsalicylic acid solutions and acetic acid solutions, using as our model VX2 carcinoma cells in suspension. The aim of this study is to analyze the effects of bicarbonate acetylsalicylic acid solution and aqueous acetic acid solution on VX2 carcinoma cells in suspension in vitro, and to determine whether these solutions cause neoplastic cell death.

Methods

In order to investigate the *in vitro* effects of the studied solutions on tumor cells in suspension, each test was performed in triplicate. After removing and macerating the tumor-bearing livers of rabbits, we produced the tumor cell suspension, from which a suspension containing 10^7 cells was drawn. This was then incubated at 37°C in two different concentrations of acetic acid, (2.5 and 5%), acetylsalicylic acid (2.5 and 5%), as well as in saline solution. Each solution was used in a volume of 0.4 ml. Every 5 minutes during the incubation, the viability of tumor cells (in %) was determined by the trypan blue test and analyzed under light microscopy.

VX2 Carcinoma tumor model

As our experimental model, we used VX2 carcinoma in rabbits. The stock cell suspension was generously provided by The Harvard Institutes of Medicine in 1999. The *in vivo* tumors have been maintained by the intrahepatic injection of 10^7 cells every 7 days in receptor rabbits.

Cell viability test: trypan blue exclusion technique

In order to determine the viability of tumor cells, 0.1 ml of 0.2% trypan blue was added to the tumor cell suspension in each of the solutions (0.9 ml each). Subsequently, a drop of suspension was placed into a Neubauer chamber and the percentage of living tumor cells was calculated. Cells that showed signs of staining were considered to be dead, whereas those that excluded trypan blue were considered viable.

Test solutions

The drugs tested were acetic acid (aqueous solution) at 2.5% and 5% and acetylsalicylic acid. In order to obtain the desired concentrations, 500 mg of acetylsalicylic acid was dissolved in 10% sodium bicarbonate (20 ml and 10 ml, respectively), forming bicarbonate acetylsalicylic acid. The solutions (0.4 ml each) were prepared 2 minutes prior to use. As a control, saline Solution was used in an equal volume (0.4 ml).

Statistical analysis of the results

In this study, the relative frequency (in %) of living cells was determined based on the mean values obtained in the repetitions.

Results

The mean results obtained through trypan blue exclusion are shown in Table 1 and Figure 1, where we observe the viability percentage of tumor cells incubated in the various test solutions or in saline solution (control group). Evaluations were performed every 5 minutes.

Figures 2a and 2b show the general appearance of tumor cells under light microscopy and figures 3a and 3b under electronic microscopy at the end of the 30-minute incubation in saline solution and acetylsalicylic acid solution, respectively.

TABLE 1 - Mean cell viability (%) after 30-minute incubation of tumor cells (10^7) in saline solution or in different concentrations of either acetylsalicylic acid (ASA; 2.5% and 5%) or acetic acid (AA; 2.5% and 5%)

SOLUTION →	SALINE	ASA 2.5%	ASA 5%	AA 2.5%	AA 5%
0 minutes	99%	99%	98%	98%	99%
5 minutes	98%	83%	78%	76%	67%
10 minutes	97%	78%	70%	68%	60%
15 minutes	97%	59%	54%	52%	49%
20 minutes	97%	28%	19%	32%	23%
25 minutes	96%	12%	4%	10%	7%
30 minutes	96%	0%	0%	0%	0%

In Figure 2a, the cells are translucent and show no staining, implying that these are viable cells. In Figure 2b, the cells are intensely stained with blue (dead cells). The staining observed in the incubation with 5% acetylsalicylic acid solution was identical to that observed with 2.5% acetylsalicylic acid solution and with both (2.5% and 5%) acetic acid solutions.

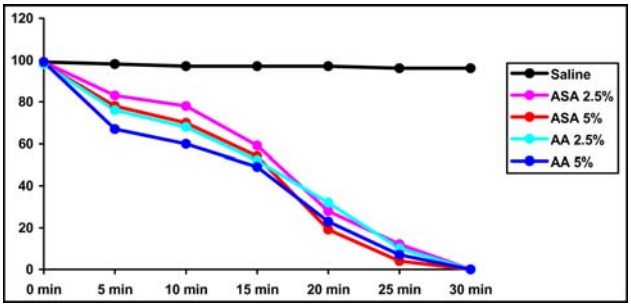

FIGURE 1 - Mean percentage of viable tumor cells after 30 minutes of incubation in saline solution, acetylsalicylic acid (ASA; 2.5% and 5%) and acetic acid (AA; 2.5% and 5%)

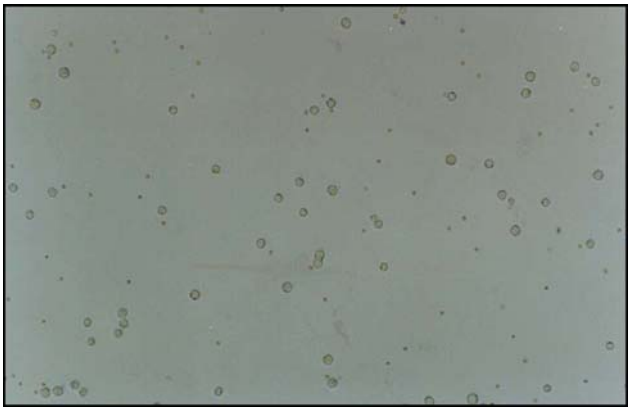

FIGURE 2a - Appearance of tumor cells under light microscopy after 30 minutes of incubation in 0.4 ml of saline solution and submission to trypan blue exclusion cell viability test. Viable cells (translucent) do not stain. x200

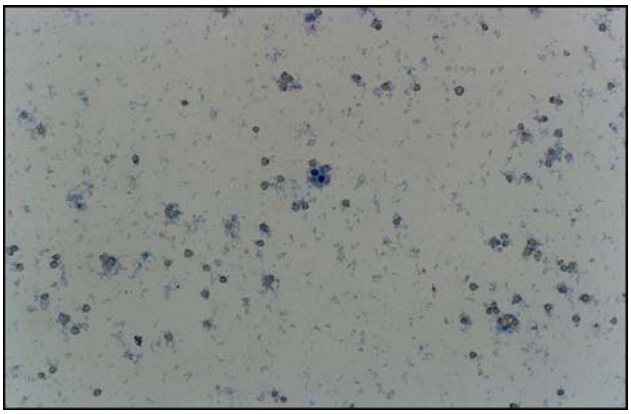

FIGURE 2b - Appearance of tumor cells under light microscopy after 30 minutes of incubation in 0.4 ml of 5% acetylsalicylic acid (ASA 5%) and submission to trypan blue exclusion cell viability test. Dead cells are intensely stained. x200

Discussion

In the quest for improved diagnostic techniques, tumor detection and prognoses, the VX2 tumor has long been employed in studies involving tomography[4,5], nuclear magnetic resonance[6,7], PET scans and chemotherapy[8-13], as well as in studies of other treatment modalities such as chemoembolization[14] and radiofrequency[15,16].

In the literature, the concentration of tumor cells classically used to maintain a tumor is one equal to or higher than 10^7 cells/ml. In our study, the same concentration (10^7 cells) was used in order to evaluate the *in vitro* effect. In our previously cited 1996 study, we used a solution composed of acetic acid, glycerin and phenol in the treatment of Ehrlich ascites tumor cells[4]. We observed that, *in vitro*, the solution destroyed tumor cells and increased the number of inflammatory cells.

The results were dose-dependent. However, we also observed a high death rate in the mice studied. This was attributed to the toxicity of the drugs in combination, since all three have known toxic effects. Therefore, it seemed appropriate to test one or more of these components separately in the livers of healthy animals and in the livers of animals with tumors. Taking into consideration the high toxicity of phenol and the fact that glycerin is used as a vehicle for other drugs, we opted for the use of acetic acid alone. In a pilot experiment, we observed that, in fact, acetic acid has a destructive effect when introduced into the liver. We decided to study the effect of acetylsalicylic acid as well because it would afford us the opportunity to study both of its components. The acetyl component (which can be analyzed through observation of the lytic effect of acetic acid) and the salicylic component (salicylic acid and the salicylates are, traditionally, applied externally as cytolytic agents) could be individually analyzed.

The in vitro study demonstrated that both tested solutions (acetic acid and acetylsalicylic acid), in the concentrations (2.5% and 5%), cause tumor cell death. This effect does not seem to be concentration-dependent, since similar effects were observed with the different concentrations tested. Regardless of the solution employed, total cell death occurred after the same period of time (30 minutes) in all instances. We observed that, *in vitro*, the acetic acid and acetylsalicylic acid solutions have an immediate and intense effect on tumor cells, whereas no such effect was observed in the control group. In the literature, there is no data regarding the *in vitro* use of VX2 tumor cells. Therefore, we had no standard against which to compare the results obtained in the present study. These results encouraged us to investigate the *in vivo* effects on the liver of healthy rabbits and rabbits with VX2 tumors.

Conclusion

In vitro, the acetylsalicylic acid and acetic acid solutions cause neoplastic cell death.

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