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Preliminary Study the Effect of Galbanic Acid on the Efficacy of Radiation Therapy on O26 Tumor Cells Implanted In Balb-C Mice

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ABSTRACT

Introduction: In the present study we used Galbanic acid (GA) combined with radiation therapy to raise radiation efficacy. GA extracted from Ferula species and have anti-cancer properties. The purpose of this study is to investigate the effect of Galbanic acid on C26 tumor radiation sensitivity and The main challenge of this study is to deliver the drug to the tumor cells due to the hydrophobicity of Galbanic acid.

Material and Methods: 28 inbred male Balb/C mice were injected subcutaneously with 500,000 C26 cells into the right flank. The mice were divided into 4 groups including group control, radiation alone (R), GA alone (GA), and both radiation and GA (R+GA). Life span, tumor growth and weight of the mice were evaluated. All analyzes were performed by the 26th version of SPSS and P value of less than 0.05.

Results: The median lifespan in group R+GA was 28 days, which was longer than 20 days in group R, 22 days in group GA, and 16 days in the control group. The lifespan of the GA+R group was significantly higher than the other groups ($P<0.05$). The percentage of growth delay in group R+GA was 78%, while in group R and in group GA it was only 42% and 47%, respectively.

Conclusion: The results showed that the lifespan and tumor growth rate of the mice that received combined treatment was longer and lower, compared to the two groups that received GA or radiation alone.

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Introduction

Cancer is one of the most common causes of death worldwide and its treatment is a challenge. Radiotherapy is one of the main cancer treatment modalities, but encounters tumor resistance. Chemo-radiotherapy, a combination of radiation and chemo-drugs, may be a clue to overcome this problem. Chemo-drugs have been exploited to radiosensitize cancerous cells via different mechanisms. Ceasing tumor cells in the G2/M phase, where they are much vulnerable to radiation damage is one mechanism. Hampering DNA repair capability and mimicking the effect of oxygen for hypoxic cells are the two other mechanisms. Furthermore, applying chemotherapy concomitantly or asynchronously with radiotherapy causes a massive loss of the cells that renders the reopening of previously closed capillaries and re-oxygenating of the hypoxic cells which are resistant to both radiation and drugs [1, 2]. Besides, some drugs prevent molecular pathways leading to proliferation, angiogenesis, or anti-apoptotic molecule expression. However, owing to the toxicity of radiation and chemo-drugs, serious side effects may occur in normal tissues. Many efforts have been made to solve this problem. Thanks to the advances in radiotherapy, small radiation fields are employed nowadays to irradiate tumors with only a narrow margin around the tumor and protect normal tissues. Emergence of antibody-Drug Conjugates has been another solution [3]. Antibody-Drug Conjugates are attached to monoclonal antibodies and target cancerous cells with higher expression of antigens and exclude normal cells.

Another approach has been utilizing natural products, with less toxicity than chemical agents, to enhance radio-sensitization [4]. Natural extracts from plants and dietary resources have played a crucial role

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in traditional medicine and been used for millennia to prevent and treat several diseases. Some of them have gained immense attention in recent time to treat cancer. They have wide safety profiles and able to target heterogeneous populations of cancer cells [4, 5]. Galbanic acid (GA) is a herbal product belonging to sesquiterpene coumarins from Ferula species (Apiaceae). *F. assa-foetida and F. szowitsiana*, two kinds of Ferula species, are rich sources of GA [6]. GA has shown various biological properties including cancer chemopreventive, anti-coagulant, antiviral, and anti-leishmanial activities. In addition, its activity against inflammation has been demonstrated. GA has also exhibited anti-cancer effects including anti-proliferation and anti-angiogenesis properties, the capability of inducing apoptosis, and inhibition of drug efflux [7, 8].

The cytotoxic effects of GA have been demonstrated for a wide range of cancer cell lines [7, 8] but not for normal human endothelial cells [9, 10] and normal tissues [11] Kim et al [12] showed GA significantly enhanced the apoptotic activity of Tumor Necrosis Factor (TNF) related apoptosis-inducing ligand (TRAIL) through inhibition of multidrug resistance 1 (MDR1) expression, activating caspases, and upregulation of death receptor 5 (DR5). Nik et al[9] also showed that GA comparably with Bevasizomab, a known inhibitor of angiogenesis, significantly limited normal neovascularization in a chicken chorioallantoic membrane angiogenesis model. According to the above properties, it was predicted that using GA with radiotherapy enhances the treatment outcome. Therefore, in the present study GA was administered concomitantly with one-session radiotherapy and its effects on the growth of the tumors consisted of C26 colorectal cancer cells, subcutaneously implanted in Bulb/C mice, and the mice's lifespan were assessed. The purpose of the present study is to investigate the effect of Galbanic acid on increasing the efficiency of radiotherapy of tumors containing c26 cells in Balb C mice.

Materials and Methods

Cell culture

The C26 cell line (C53, murine colon carcinoma cell) was purchased from the Pasture Institute (Tehran, Iran). Cells were maintained in Roswell Park Memorial Institute Medium (RPMI-1640), supplemented with 10% fetal bovine serum (Gibco), 100 µg/mL streptomycin (Sigma), and 100 units/mL penicillin (Sigma), in the standard humidified environment of 37°C and 5% CO_2.

Animal study

In this method, 40 Balb/c male mice in the weight range of 15.2-23.2 grams and aged 4-6 weeks (12 for pilot experiments and 28 for the main experiment) were used (purchased from Pasteur Institute). During the experiment, the mice were kept in polycarbonate cages under standard conditions (light cycle of 12 hours of light and 12 hours of darkness, free access to water and special compressed food, temperature of 25°C and humidity of 45-55%).

In order to tumorize the mice, first we shaved the hair of the part of the mice's body where the injection is to be done and disinfected with 70% alcohol. Then we counted the C26 cells (colon carcinoma) that we separated from the bottom of the flask with trypsin. After counting, we dissolved the cells in the appropriate volume of sterile Phosphate buffered saline (PBS) to be ready for injection. Before injection, we anesthetized the mice with 100 mg/kg ketamine and 10 mg/kg xylazine so that the cells did not move during the injection. We injected 500,000 cells in 100 microliters of cells into each mouse using an insulin syringe under sterile conditions. To avoid contamination, we did not put the mice in the box with wood shavings for 24 hours. After 24 hours, we changed the animal housing boxes again and added wood chips to create a suitable substrate. After 7-10 days, the tumors appeared as small lentil seeds.

Preparation of drug suspension

In order to make a Galbanic acid solution with a concentration of 50 mg of Galbanic acid per 10 ml, we first dissolved 50 mg of Galbanic acid powder (Golexir pars, CAS number: 3566-55-0) in 0.5 ml of twin 80 and then made up to 10 ml with distilled water. The solution was then placed in a sonicator to mix well and form a suspension. Each mice received 50 mg / kg Galbanic acid at each treatment. For this purpose, according to the weight of each mice, an appropriate volume of suspension (1 ml per 100 g) was fed to each mouse by gavage.

Grouping mice

The mice were divided into 4 groups as follows:

The control group did not receive medication or radiation.

The drug group (GA) received Galbanic acid and were not irradiated.

The radiation group (R) received radiation.

The radiation and drug group (GA+R) received Galbanic acid and were irradiated.

Treatment

When the tumor appeared in mice, we started Galbanic acid treatment in the GA group and the GA+R group by gavage. Drug treatment was continued 6 times for 2 weeks (3 times a week). It should be noted that the mice in the radiation group and the control group that did not receive the drug received the solvent 6 times for 2 weeks by gavage.

When the tumor size reached 100 mm^3, the tumors of the mice in the R group and the GA+R Group were exposed to 6Gy radiation. Irradiation was performed by a linear accelerator (Compact Elekta, Stockholm, Sweden). Prior to irradiation, the mice were anesthetized with 100 mg / kg ketamine and 10 mg / kg xylazine to prevent them from moving during irradiation. The Mice

were in AP position and Irradiation at a rate of 2 Gy / min, at SSD = 100 cm, was performed only on the tumor (Figure 1). Irradiation was performed in such a way that the middle of the tumor received a dose equivalent to 6 Gy in one fraction.

The weight of mice and their tumor size were measured every other day. Also, their general health was monitored until death. The basis for measuring the lifespan of mice in different groups was the day of their death, or tumor volume reaching 1000 mm^3, or weight loss of more than 20%, or the number of mice being healthy and unable to feed, in which case Ethical standards in animal research were killed [13]. To calculate the tumor volume, we measured three diameters of the tumor with calipers and then used formula 1 [13]:

$$\text{Tumor volume } (mm^3)=(\text{length}\times\text{height}\times\text{width})\times 0.52 \quad (1)$$

Also, we calculated various survival indices for all groups, including the percentage of increased lifespan of mice (%ILS), Time to end point (TTE) and percentage of Tumor growth delay (%TGD) for all treatment groups. TTE quantity represents the Time to reach the End Point, which here is the tumor volume size reaching 1000mm^3. To obtain TTE, we take the logarithm of the tumor volume on the day when the tumor size reached 1000 mm^3 and also from the three measurements before reaching the size of 1000 mm^3. After that, we drew the logarithmic curve per time unit and through the equation of the line obtained from the following formula 2, we obtained the TTE for each mouse in the group [13].

$$TTE = \frac{\log 1000 - intercept}{Slope} \quad (2)$$

Then we average the TTE value of the mice in the group and calculate the average TTE of that group [13].

The amount of TGD represents the delay in tumor growth and is obtained as follows the formula 3:

$$TGD\% = \frac{\mathrm{T(day)-C(day)}}{\mathrm{C(day)}} \times 100 \quad (3)$$

In the above equation, T represents the average TTE of the treated group and C represents the average TTE of the control group.

The amount of ILS represents the increase in life expectancy and is obtained as follows the formula 4 [13].

$$ILS\% = \frac{\text{Treatment under the mean survival group}}{\text{control group mean survival}} - 100 \quad (4)$$

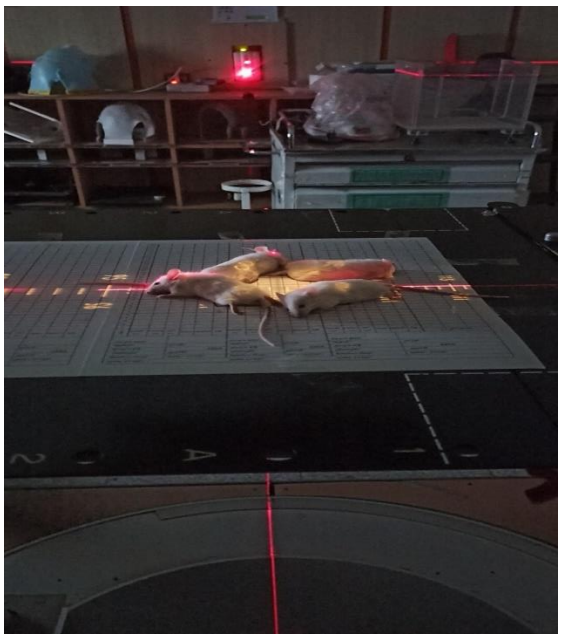

Figure 1. The tumors of the mice in the Radiation group and the Galbanic acid +Radiation Group were exposed to 6Gy radiation. Irradiation at a rate of 2 Gy / min, at Source to skin distance (SSD) = 100 cm, was performed only on the tumor

Statistical analysis

Normality test (Kolmogorov-Smirnov), one-way analysis of variance, Tukey test, and Kaplan-Meier test were used for statistical analysis of the relative volume of the tumor and the lifespan. Comparisons were made at 95% confidence level with a P value of less than 0.05. All analyzes were performed by the 26th version of SPSS.

Results

As can be seen in Figure 2, in the present study, we did not see a weight loss of more than 20% of the initial weight in any mouse. Therefore, none of the mice in this study were killed due to weight loss.

As Figure 3, the relative volume of the tumor is smaller in the radiation + drug, radiation alone and drug alone groups, respectively, than the control group.

The average tumor volume of the GA+R group was significantly lower than the control group from the day 6 ($P<0.05$). The average tumor volume of the R group, from the day 14, and the average tumor volume of the GA group, from the day 16, were significantly lower than the control group ($P<0.05$). There was no significant difference between the three test groups. However, it was observed that the GA+R group had the slowest tumor growth.

Based on Table 1, the GA+R group had the highest Time to endpoint (TTE) value (27.3 days). R, GA, and control groups had the TTE values of 22.6, 21.8, and 15.3 days respectively. The same trend was observed in other survival indices as, Tumor growth delay percentage (% TGD) and Lifespan percentage (ILS %) were the highest in the GA+R group. (Table 1).

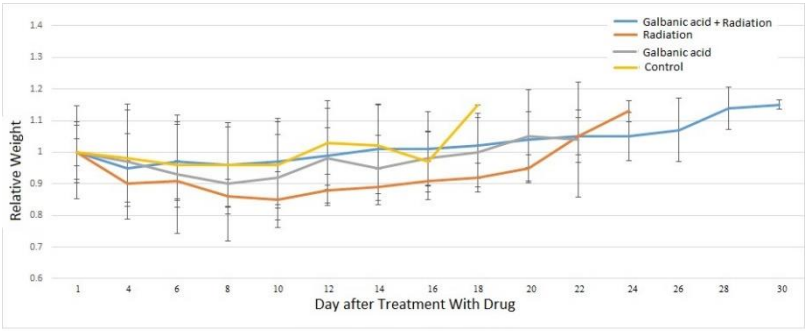

Figure 2. Graph of relative weight of mice. None of the groups experienced a weight loss of more than 20% compared to the first day

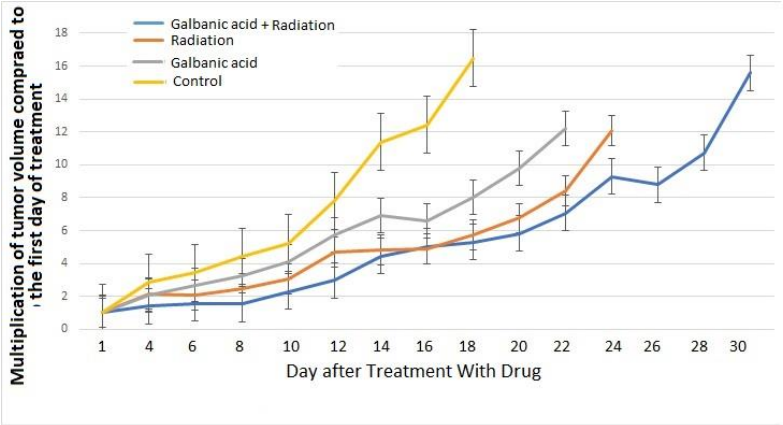

Figure 3. The relative volume of the tumor against time

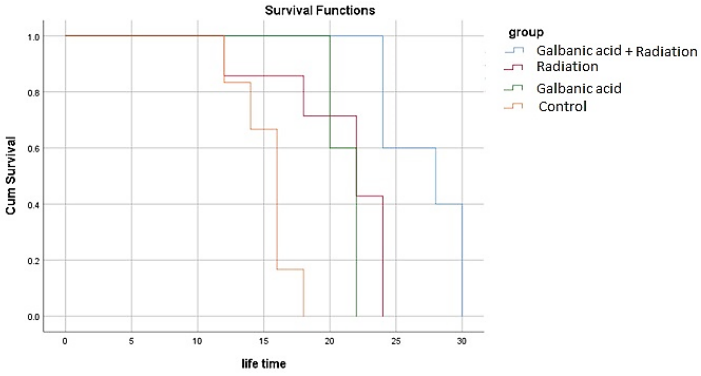

Figure 4. The survival of the mice against time. Day zero in the figure indicates the day of starting drug treatment

Table 1.the survival indexes of different groups

Group	TTE+Sd	TGD%	ILS%	Mean (day)±Std	Mode (day)
Galbanic acid+Radiation	27.3± 2.1	78	77.7	27.2±3	28
Radiation	22.6±5	47	30	20.2±4.6	20
Galbanic acid	21.8±1.4	42	38.5	21.2±1	22
Control	15.3±1.4	-	-	15.3±2	16
Time to endpoint (TTE), Tumor growth delay percentage (TGD%), Increase in life expectancy (ILS)					

In addition, according to the statistical analysis, the lifespan of the GA+R group was significantly higher than the other groups (P <0.05). The lifespans of groups R and GA were also significantly higher than the control group (P <0.05) (Figure 4).

Discussion

In the GA+R group, TGD and TTE were more than in the control, R, and GA groups (Table 1). In addition, the slope of the tumor growth diagram in the GA+R was lower than in the other groups (Figure2). These observations suggest that combination therapy (GA+R)

is superior to radiation therapy alone or drug alone in inhibiting tumor growth**.** On the hand, there was no significant difference between the three groups of combination therapy (GA+R), radiation alone and drug alone in the tumor volume on different days., However, in animal studies the main criteria showing the rate of tumor growth are TGD and TTE, which were higher in group GA+R. Therefore, the lack of significant difference in tumor volume on the days after treatment can be attributed to the edema or necrosis in tumors.

The lifespan of the animals was also evaluated and it was shown that the lifespan of the mice in group GA+R was longer than the other groups (Figure 4), which again shows the superiority of combination therapy over radiation alone and drug alone .

Besides, monitoring the weight of the mice revealed no weight loss in the R+GA group, which indicates the tolerability of the combinatorial therapy used in the present study. Previously, Afsharzadeh et al [11] found that neither free galbanic acid nor pegylated galbanic acid pegylated with liposomes had a deleterious effect on healthy tissues such as heart, lungs, etc.

Although it is the first time that radiotherapy has been combined with GA treatment, other researchers have combined chemotherapy with GA in several studies. In a study carried out by Nik et al [9] pegylated liposomal doxorubicin (PLD) was used accompanied by GA to treat tumors implanted in Balb/C mice. They observed that the tumor growth rate in the group that received both PLD and Galbanic acid was lower than in the group which was treated with PLD alone. Nik et al [13] observed a similar result when they used GA in combination with Doxorubicin. In harmony with our result, they showed that the lifespan of the mice was longer in the GA+ Doxorubicin compared to the group which was treated with just Doxorubicin.

The better response of the tumor to combinatorial therapy can be attributed to the anticancer properties of galbanic acid. The anti-angiogenic properties of galbanic acid have been proven in many studies [14, 15] Afsharzadeh et al [11] observed that galbanic acid prevents angiogenesis. Eskandani et al [12] also observed that galbanic acid reduced *Hypoxia-inducible factor 1-alpha* and Hypoxia-Inducible Factor 1βeta molecules, which increase Vascular endothelial growth factor **(**VEGF **)** expression and consequently angiogenesis. The anti-angiogenic properties, apart from any interaction with chemotherapy or radiation, make it possible for galbanic acid to prevent tumor growth and increase the effectiveness of radiation or drug therapy.

In addition to anti-angiogenic properties, galbanic acid has other anti-tumor features, which make it more convenient to be combined with radiotherapy/chemotherapy. For example, it has been observed that Galbanic acid via decresing the half-life of Epidermal Growth Factor (EGF) molecules [15] prevents tumor cell proliferation. Moreover, Ove et. al. [16] observed Galbanic acid not only decreases the expression of anti-apoptotic proteins such as B-cell lymphoma 2 (BCL-2), B-cell lymphoma-extra large (BCL-X) , and Myeloid leukemia 1 (MLC1), but also increases the level of pro-apoptotic proteins (Bax and caspase 9). AS MCL1 molecules are involved in the spread of cancer stem cells [13] galbanic acid, by decreasing MCL1 molecules, may also decreases the number of cancer stem cells. But, it remains to be investigated in the future studies.

Directly increasing radiation sensitivity by methods such as impairing Deoxyribonucleic acid (DNA) damage repair mechanisms, sensitizing DNA structures, killing S-phase-resistant cells, or ceasing tumor cells in the G2/ M phases, requires drug delivery to the tumor before irradiation. Therefore, as in the present study, we did not study the distribution of the drug in the blood circulation and in the tumor, we cannot conclude that Galbanic acid has directly increased the radio-sensitivity of the tumor. Nevertheless, we can suggest that the mentioned mechanisms, anti-angiogenic, anti-proliferation, and proapoptotic properties of Galbanic acid, may have improved the outcome of radiation therapy.

Some researchers have tried to increase GA solubility in water. Nik et al [9] used liposomal pegylated GA rather than free Galbanic acid to elongate GA presence in blood circulation and decrease GA toxicity. In addition, they attached an integrin ligand to the liposomal pegylated GA to increase its accumulation in the tumors. Afsharzade et al [11] chose another approach to raise the GA delivery to the tumor. They used poly (D, L-lactide)–polyethylene glycol nanoparticles as a carrier for GA and compared their delivery to tumors with the delivery of free GA. The results showed a better accumulation of nanoparticles in the tumor cells and a better treatment outcome. They observed that nanoparticles decreased tumor growth more effectively than free GA. In the present study, we used free GA; however, the superiority of combinatorial treatment was yet obvious. Therefore, it can be predicted that a combination of radiotherapy with a non-free form of GA may promote the treatment result more effectively.

Conclusion

The results of this study showed that radiotherapy combined with GA treatment improved the treatment outcome. In the present study, the tumors received only 6 Gy radiation in one session. However, in future studies, the tumors should be treated with adequate radiation dose. Hence, it is possible to judge whether combinatorial treatment leads to a complete cure in the mice. In addition, the appropriate form of GA should be used rather than free GA and should be monitored in the tumor and blood circulation to determine the appropriate time schedule for feeding or injecting the mice with GA. Besides, in vitro studies can investigate the therapeutic mechanism of Galbanic acid when combined with radiation and answer the question of whether GA radio-sensitizes the cancerous cells.

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