Treatment of Colon Carcinoma Tumors in Balb/c Micethrough the Electrolysis Method: The Effect of Dose Distribution

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Introduction

Currently, common methods of treatment and control of cancer include surgery, radiotherapy and chemotherapy, which are used individually or combined. However, there are some cancers that are not treatable through conventional methods and need using complementary treatments [1, 2]. Hepatocellular carcinoma and colorectal liver metastases are among these diseases. Surgery is considered the only effective treatment method to treat these cancers. But unfortunately, some factors such as multiple tumors, closeness and invasion of cancerous cells to the main artery, magnitude of tumor and the patient's extreme weakness make only 20% of patients suitable for surgery [1, 3-5]. A new approach to eradicate tumors which has been seriously considered and evaluated recently is Electrochemical Therapy-ECHT or electrolysis therapy which is simple, cheap with minimal side effects and it is also safe and has less SIRS-systemic inflammatory reactions than the other new physical techniques such as Cryotherapy, Laser interstitial thermotherapy, radio frequency ablation and High-intensity focused ultrasound treatment [1, 6, 7]. This method can be also used in the vicinity of main arteries and totally destruct the surrounding cancerous cells without damage to the arteries, while it is not possible in the mentioned method [1, 8]. In this technique, under the influence of potential and applied electric field, the tumor gets polarized and subsequently electrochemically analyzed and the tumor gets necrotized and disintegrated under the effect of some factors such as extreme changes in pH, induced electric field, produced toxins and other possible factors, including immune system stimulation [4, 9, 13-19].

This method is used in treatment of inoperable tumors as well as superficial, primary visceral and metastatic tumors and promising results have been reported [10]. Electrolysis therapy was confirmed in China in 1989 and within a decade, 15,000 patients with various malignant, benign superficial and visceral tumors were treated [11, 12]. Despite the promising results, this method have not still obtained
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a clinical license in the West; because there are still some ambiguities regarding tumor destruction mechanisms and optimal conditions for interfering factors such as electrical parameters (current, electrical dose, current density, etc.) and electrode polarity and arrangement [12, 13]. Accordingly, the present study examines the effect of electrolysis as well as the effect of dose distribution -which seems to flow better with the increasing number of current supplies- by using a horizontal sixth electrode arrangement at base and top of the tumor with direct electric current of 5 mA on colon carcinoma tumor model in Balb/c mice.

Materials and Methods

This experimental study was conducted in the Cancer physical therapy laboratory of Medical Physics Research Center in Mashhad University of Medical Sciences. In order to investigate the effect of the electrolysis and dose distribution, after a consultation with a statistic and literature review, 60 mice were selected [9, 21] that were randomly classified into 4 groups (control groups 1 and 2 each containing 10 animals, treatment groups 3 and 4 each containing 20 animals). Group 1 (without electrode placement in tumor) and group 2 (placement of 6 electrodes in two rows in the tumor for 45 minutes without applying current and dose) were respectively considered as control and main control group. The control group 2 was considered to eliminate the interfering and unpredictable effects of platinum electrode in the incidence of necrosis and thus, it was called the main control group. In Group 3, the current 5mA was applied to the 6 electrodes by a current supply, while in group 4, three current supplies were used each of which was separately connected to a pair of anode and cathode electrode to apply dose. It is worth noting that in the test groups, the electrodes located at the base of tumor were connected to the negative pole of the device and played the role of cathode and the electrodes located at the top of tumor were connected to the positive pole and played the role of anode.

Culture and proliferation of cells: CT26 cell line derived from colon carcinoma in Balb/c mice was purchased from Iran Pasteur Institute. It is worth noting that during performing this project, this line was the only cell line available in the country that was able to create unlimited fast growing tumor in Balb/c mice (figure 1). Then, tumor cells in sterile flasks in RPMI-1640 medium containing 10% FCS and streptomycin antibiotics (100 g/ml) and penicillin (100U/ml), were cultured and amplified as suspension in the incubator of 37°C containing 5% carbon dioxide.

After two to three days of growth and proliferation, the cell covered the flask bottom as monolayer (figure1-A). The cells were separated from the flask bottom by trypsin-EDTA and in sterile conditions under the hood. Having been trypsinized, the cells were counted and the percentage of viable cells was determined using Trypanblue method and Neubauer lam and cell suspension was prepared with concentrations of 5x105 viable cells per ml in Hanks solution.

Figure 1. A) CT26 cell line derived from colon carcinoma of Balb/c mice B) Subcutaneous model of colon carcinoma tumor of Balb/c mice; group 1, 70 days after injection of tumor cells, rat weight 20gr, tumor weight 30gr C) View of non-necrotic colon carcinoma tumor cells in Balb/c mice;H&E staining; 400 times magnification.

Creation of animal tumor model: tumor model in Balb/c mice (6-8 weeks old, weighing 20-28 g) which were purchased from Pasteur Institute of Iran was induced. The animals were kept in standard conditions of laboratory in special cages in a separate room at temperature 23±2°C and 65 percent humidity, at normal day-night cycle and on a standard diet (rat food, KhorasanJavaneh Company). Then, each rat was injected with 5x105 viable cells in 100μlHanks solution. Animals were injected subcutaneously in the right flank. From the day when tumor volume reached about 100±20mm³, their dimensions [small diameter (a), large diameter (b) and tumor thickness (c)] were daily measured using digital caliper and tumor volume (V) was calculated through the relation $V = \pi/6 (a.b.c)$ [9].

Animal Anesthesia: Before placing the electrodes in the tumor and applying the current, the animal was anesthetized by intraperitoneal injection of 10 mg/kg xylazine (made by BehyarSaman pharmaceutical company), 2%Xylazine and Ketamine 50 mg/kg (Ketamine 150 mg/ml; Rotexmedica, GmBH). Also sterile eye drops were used to avoid dry eye in the animal during general anesthesia [21].

Constant current electrolysis system: In order to apply the desired electrical dose, a constant current electrolysis system was used, which was designed and made in Medical Physics Research Center of Mashhad University of Medical Sciences. This current supply is able to apply electrical constant current with an accuracy of 0/1 mA to the varying ohmic resistance of the tissue during the treatment (tissue resistance during electrolysis varies over a few thousand ohms).

The system has four separate output channels with separate monitors of voltage (V), current (mA), time (S) and charge (C). To enhance safety and prevent possible risks arising from the increase of electrical current intensity, this system has current limiter and adjustable voltage and contains an alert system indicating the end of treatment and the voltage and
current exceeding from the adjusted range for the patient as well as short circuit during the treatment.

Electrodes: Electrodes were made of platinum wires (MERK) with very high purity (99/99%) with a diameter of 0/7 and a length of 20 mm. For easier entry of electrode to the tumor, they were sharpened and sterilized before each treatment session [21].

Treatment methods and groups: The treatment of the tumors was performed when their volume reached 340±30 mm³ and after the animal preparation. This stage includes the control of animal weight, removal of the tumor hairs and surrounding hairs, measuring tumor dimensions and calculation of duration and amount of the applied dose according to the tumor volume and base dose, intraperitoneal injection of anesthetic and placing electrodes in the tumor. Treatment duration was calculated according to Ampere’s law (q = It) and the applied dose (q) and electric current (I). Based on the previous conducted studies, the maximum tolerable current (depending on size and weight of animal) was 5 mA that was selected in this study [21]. In addition, the electrode array in the tumor was selected as horizontal and parallel with the surface of rat’s body with arrangement of two triplet rows on the base and top of the tumor. According to the proper efficiency of electrolysis and small size and low weight of the animal and therefore high sensitivity, the treatment was performed in only one session [9, 21]. In order to prevent shock in mice, the current was gradually reached to 5MA or vice versa to zero, in one minute at the beginning and end of treatment [12]. After the treatment, the animal was quarantined and was kept separate.

After killing the animal, the tumor with a margin of 0/5 cm was removed and placed in 10% formalin buffer solution. The samples were put in the fixator solution remained after cutting and preparing paraffin blocks from superficial and deep sections for 48 hours and were stained with hematoxylin and eosin. Totally, several microscopic slides were prepared from each tissue samples. Microscopic slides prepared from the samples were examined by pathologists. In order to estimate the extent of necrosis in the samples, different tissue sections in slides of each sample were examined by Olympus BX40 microscope and the average necrosis was determined in each sample the estimating the amount of necrosis in each section. Statistical analysis: Considering non-normality of changes in volume of tumors and survival of animals, “Mann-Whitney U” test was used to compare changes in volume and the computational method of Kaplan-Meier and log rank analysis to evaluate an increase of necrosis started severely, so that 5 days after the treatment, more than 80 percent of tumor volume has been reduced and only an ulcer-like lesion were seen in the treated area in all tumors of the two treatment groups. Relapses usually occurred up to 3 weeks after the treatment and no relapse after this period indicated complete response. In that case, within a month after the electrolysis, the treated area in these animals completely recovered and only a small fibrous scar remained in the area which was covered by hair. Figure 2 shows the tumor changes caused by electrolysis in a tumor model treated with a dose of 20C/cm³ in the cathode base polarity using three current supplies.

Results

In the initial pilots with one current supply, dose distribution in tumors is not uniform due to the non-uniform electric current in the electrodes which is caused by different electrical resistance in their path, and even several cases of recurrences were seen, which were probably caused by this phenomenon. To fix this problem, three current supplies were used for supply of each pair of electrodes (anode and cathode) and through this mechanism; the current in the electrodes became identical, despite different tissue resistance on the pathway of each of them. Physical and pathological studies also indicated the more uniform dose distribution with three current supplies. The purpose of considering the group 4 was also the same results.

Qualitative observations after electrolysis: Immediately after the treatment began, the electrochemical interactions started in the tumor, and especially around the electrodes and gas bubbles were formed and released around the electrodes, which are supposed to be chlorine and oxygen in anode, and hydrogen in cathode [8,11,23,24]. Further, severe physical changes occurred in the tumor (color change and shrinkage of tumor and edema) due to the electrochemical changes and processes such as electroosmosis, which will be particularly revealed by over time. After electrolysis, the reduction of size and increase of necrosis started severely, so that 5 days after the treatment, more than 80 percent of tumor volume has been reduced and only an ulcer-like lesion were seen in the treated area in all tumors of the two treatment groups. Relapses usually occurred up to 3 weeks after the treatment and no relapse after this period indicated complete response. In that case, within a month after the electrolysis, the treated area in these animals completely recovered and only a small fibrous scar remained in the area which was covered by hair. Figure 2 shows the tumor changes caused by electrolysis in a tumor model treated with a dose of 20C/cm³ in the cathode base polarity using three current supplies.
Changes in tumor volume: Changes in tumor volume in the groups 72 hours before and after the electrolysis can be seen in diagram 1. The significant points in the above diagrams include severe and significant decrease in tumor volume in treatment groups compared to control groups ($p < 0.001$). However, the reduction in groups 3 and 4 is not significant ($p > 0.05$). In diagram 2, changes in tumor size are seen up to 40 days after treatment (due to the extended onset of death after 40 days, especially in control groups and its effect on tumor volume changes, the chart is shown up to this time). While the tumors in groups 1 and 2 respectively had rapid growth with DT: Doubling Time of $6.1 \pm 3.9$, and $5.9 \pm 2.9$ days, electrolysis significantly managed to control and delays their growth in treatment groups ($p=0.000$).

The response rate to an electrolysis therapy session: In the two control groups, the tumors grew rapidly and without regression or even stop according to diagram 2, and even in a few mice, tumors reached the weight of more than 30 grams, which was remarkable compared to the animal weight (20 g), and thus all mice died of the complications caused by cancer (Fig. 1-C). Whereas, in group 3, 40 (8.20) percent and in group 4, 60 (12.20) percent complete responses were seen after one treatment session. Remarkably, no complete response was observed in the other tumors of these two groups, but the destruction of more than 80% was seen, which is in fact partial response. The comparison of treatment groups and control groups showed that the complete response in group 4 ($p=0.002$) and group 3 was significant ($p=0.029$) compared to the control groups. Also, although complete responses in group 4 were more than group 3 (20% more), the difference was not dramatically significant.

The average survival of animals in control groups 1 and 2 was respectively recorded $55 \pm 17$ and $49 \pm 14$ days. While in the treatment groups 3 and 4, it was respectively $81 \pm 56$ and $101 \pm 57$ days assuming the worst condition (death of all animals after 150 days), and it would definitely increase more by increase of the follow-up period, because the animals remaining in the end of study were quite healthy, wholesome with normal weight. Diagram 3 shows the changes in cumulative survival in different groups.

It has been revealed that while the cumulative survival percentages within 100 days after treatment have reached zero percent in control groups 1 and 2, it is respectively 35 and 55 percent in groups 3 and 4 at the end of 150 days. Data analysis through log rank test showed a significant increase in survival of group 4 compared to the control groups ($p=0.02$). In the survival results analysis, we also observe that the results of group 4 are better than that of group 3, although they are not significant. One death was seen in group 3 (127 days after treatment) and one death in group 4 (94 days after treatment) in the treated animals, the cause of which was not identified.
through pathological examination. These animals had tangible weight loss.

The results of pathological studies Microscopic examination: In the microscopic examination of the prepared tissue slides, tumors were composed of cells with spherical, elliptical, or spindle-shaped nuclei, low to moderate cytoplasm of pink to purple (acidophil) and prochromatin and basophilic nuclei and some of them had specific nucleolus. The cells had no clear tissue pattern, sometimes it has irregular short cross over stripped pattern with no clear cellular and tissue differentiation in them (Fig. 1- B). Figure 3 shows the view of a tumor nodule created in the hypodermal area with specified limits and no necrosis.

**Figure 3.** A tumor nodule created in the hypodermal area, with specified limits and no necrosis, H/E staining, 40x magnification

Pathologic findings of the treated samples: the necrosis created in the tumors was coagulation necrosis the various stages of which from primary to late stage of recovery of necrosis area were observed. Although in many cases, necrosis was observed in the center of tumors expanding around, in some cases necrosis was more obvious especially in its early stages at bottom or top of tumor, where one of the electrodes was located. Coagulation necrosis occurred quite obvious with the accumulation of nuclear chromatin. Sometimes the onset of white cells was seen around the necrosis location, which in some cases was associated with liquefactive necrosis in the tumor caused by enzymatic action of white cells. Specific apoptosis was identified in some tumors, but since the evaluation criteria in this study was tissue necrosis, cell death process caused by apoptosis was not considered. In some tumors, lymph nodes which were caused by irritation of immune system and its response to the tumor padgans were observed near the tumor. Also according to the view of neutrophils in the areas around the tumor, there is evidence of the immune system stimulation by electrolysis.

Rarely, in some animals, vascular sections which were blocked through thrombosis were also observed around the necrotic tumor, in which vascular occlusion can be effective in creation of necrosis in the tumor (Fig. 4).

**Figure 4.** A thrombosed vessel in the vicinity of a destructed tumor, H/E staining; 200 times magnification

After 150 days, recovered animals while healthy and wholesome with normal weight were killed and pathology review was performed on a number of them. The results indicated the absence of tumor in the treated area in which scarred tissue with no active necrosis has been placed (Fig. 5).

**Figure 5.** Substitution of fibrosis repair connective tissue (F) for a completely disappeared tumor, in the thickness of the dermis and hypodermis; H/E staining, 200 times magnification

The average percentage of necrosis within 5 days after treatment in different groups has been shown in table 1. The results show that the average necrosis within 5 days after treatment in groups 3 and 4 was significantly higher than control groups ($p =0.029$). Comparisons of two treatment groups showed that although the average necrosis was higher in group 4 and more complete responses were also recorded in this group, but the difference was not significant. Pathological examination of a number of the treated tumor models within 5 days after the treatment showed high percentage of necrosis and sometimes even complete destruction in all of them (Fig. 1 & 6). Also in another study on vital organs (liver, kidneys, lungs, gastrointestinal tract), no evidence of metastasis was observed.

**Figure 6.** A completely necrotic tumor nodule, H/E staining, 40x magnification
Discussion

The results show that, like other physical methods of cancer treatment such as radiotherapy, for successful treatment of tumors using electrolysis, uniform dose distribution is an effective factor. During the electrolysis with a current supply, the electrodes are parallel and since the resistance to each pair of anode and cathode electrodes is different, the current passing through them is not uniform and thus the area covered by each pair of electrodes does not receive the same dose. Each pair of electrodes are connected to a current supply using three current supplies, and therefore each one would apply current of 1.66 mA (totally 5mA) without effect from the amount of electrical resistance in their way, and more complete response and higher necrosis percentage in group 4 compared to group 3 showed that this approach has led to more uniform dose distribution and thus, more likelihood of complete tumor destruction. According to the obtained results, it appear that dose distribution approach along with other parameters, including the amount of applied dose and arrangement of electrodes which have been mentioned in other studies, can affect the efficiency of treatment [9,13]. It seems that increase in number of current supplies along with proper alignment of electrodes, can help more uniform dose distribution in tumors and can be used for complete destruction of the tumor at lower time and dose. That is because when the dose distribution is not uniform, the applied dose and treatment duration will unconsciously increase to ensure destruction of all parts of tumor, which is associated with high risks such as tumor lyses syndrome which may also occur in chemotherapy. This syndrome which occurs due to an imbalance in blood electrolytes can lead to death [24]. Significant volume reduction and destruction of at least 80% of tumor volume was observed in treatment groups. This result indicates that the electrolysis has anti-tumor effects and high potential to destroy and control tumors. Researchers such as Schaefer and Samuelsson also reported the effect of increased dose on increase of necrosis [6,10,24,25]. Examining the effect of electrolysis on pig liver, Berry et al claimed that there is a linear relationship between the created necrosis and the applied dose [26]. Chou confirmed the effect of increase in dose on the significant increase of complete response and survival in Balb/c mice [21]. This study showed that the presence of neutrophils in the treated area confirms the hypothesis of stimulation of the immune system by electrolysis. A number of researchers, such as Nordenstron and Xin, have confirmed the contribution of immune system to destruction of tumors [9, 27]. Ciria believes that the immune system will be stimulated by the necrosis created by electrolysis, and will send leukocytes (including neutrophils) to the treated area and these particles contribute to the process of tumor destruction by creating acute local inflammation [9].

In the pathological examination of the tumor thrombosis, capillaries of tumor and its periphery were seen within 5 days after treatment (Fig. 10). This can avoid SIRS syndrome and be one of the reasons for complete health of the treated animals and their very rare death within 150 days after the treatment. Wemyss et al believe that when a large volume of necrosis is created, emission of the particles from necrosis in the blood can highly increase cytokines and SIRS syndrome. During the electrolysis, thrombosis of capillaries of the tumor and its surrounding narrow margins avoids the emission of particles from the necrosis in the blood and subsequently, prevents systemic inflammatory response [28]. Overall, the results obtained in this study and reviewing articles indicate that electrolysis have most conditions and features which a technique in the field of cancer treatment or control must have. It seems that low treatment costs, safety, side limited effects and high efficiency along with features, including simplicity and lack of need for advanced equipment, will cause it to play an important role in treating cancer in the future, and to be considered a supplementary method along with approaches including surgery and radiotherapy to increase efficiency of treatment and survival of patients. Perhaps the biggest problem of this method is the long duration of treatment. It seems that this problem can be adjusted by controlling and optimizing some parameters, including electrode arrangement, the dose distribution approach, polarity and the appropriate number of electrodes, and even using other techniques such as simultaneous hyperthermia [29].

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References
