Evaluation of riboflavin photochemical treatment for inactivation of HCT116 tumor cells mixed in simulative intraoperative salvage blood

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BACKGROUND: Radiation and filtration have achieved satisfactory results in inactivation or removal of tumor cells mixed in salvage blood, but some drawbacks remain. This study evaluated the inactivation on HCT116 cells mixed in simulative salvage blood by riboflavin photochemical treatment.

METHODS: HCT116 cells were added to the whole blood to simulate contaminated salvaged blood. The mixed blood was added with riboflavin of 50 μmol/L final concentration and illuminated by ultraviolet light. The samples were divided into control group and Experimental Groups 1 (18 J/cm^2), 2 (23.4 J/cm^2), and 3 (28.8 J/cm^2). An autotransfusion system (Cell Saver Elite, Haemonetics) was used to simulate the intraoperative blood salvage procedure to deal with whole blood. The apoptosis rate and tumorigenicity of HCT116 cells and the superimposed damage to red blood cells (RBCs) were evaluated.

RESULTS: The apoptosis rates of HCT116 in Experimental Groups 1, 2, and 3 were much higher than that in the control group. Tumor growth was found in the control group, but no tumor growth was found in the three experimental groups. The hemolysis rates in the three experimental groups were significantly higher than that in the control group, but much lower than the quality standard of RBCs at the end of preservation. The concentration of adenosine triphosphate in RBCs was comparable in the control and experimental groups.

CONCLUSION: Riboflavin at a 50 μmol/L final concentration and 18 J/cm^2 ultraviolet illumination can effectively inactivate HCT116 cells in salvaged blood, with minimum damage to the structure and function of RBCs, and the main quality indexes of salvaged RBCs were within the standard range.

Intraoperative blood salvage (IBS) was born in the 1960s, and it usually uses a negative pressure suction device to salvage the bleeding in the patient's body cavity and surgical field. After a series of processes, including negative pressure suction, anticoagulation, filtration, washing, resuspension, and so on, salvaged blood can be returned to the patient to increase the hemoglobin level of the patient and effectively correct anemia caused by blood loss.[1] In recent years, with the rapid development of IBS technology, it has been widely used in cardiac surgery, vascular surgery, orthopedics, general surgery, obstetrics and gynecology, neurosurgery, urology, and pediatric surgery. IBS significantly reduces the amount of allogeneic blood transfusion, greatly decreases the risk and complications of transfusion, and saves a number of blood resources.[2–4] However, due to concerns that malignant tumor cells fall off

ABBREVIATIONS: ATP = adenosine triphosphate; HE = hematoxylin and eosin; IBS = intraoperative blood salvage; LDH = lactate dehydrogenase; OD = optical density; RPT = riboflavin photochemical treatment; UV = ultraviolet.

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during the operation and pollute the salvaged blood, leading to the distant spread of the tumor through blood circulation, tumor surgery has been a relatively restricted area of IBS for many years.[5,6]

Compared with other types of surgery, tumor patients have a higher risk of bleeding, a higher chance of blood transfusion, and a greater risk of transfusion of allogeneic blood. A large number of clinical studies have shown that allogeneic blood transfusion can promote the recurrence and metastasis of malignant tumors and shorten the survival time of patients after operation.[7–9] Several studies have confirmed that tumor cells with proliferative ability can indeed be detected in red blood cells (RBCs) salvaged from tumor patients during surgery, and the return of these tumor cells to patients may lead to tumor dissemination.[10–12] To solve the problem of tumor cell contamination, the researchers used irradiation killing[13,14] or a leukoreduction filter to remove tumor cells from salvaged RBCs,[10,15,16] which make it possible to use IBS for tumor surgery.

The basic principle of inactivation of tumor cells by radiation is based on the fact that tumor cells are rich in nucleic acid and are highly sensitive to radiation damage. The destructive effect of a certain dose of radiation on DNA in the nucleus was used to kill the tumor cells mixed in the salvaged blood and make them lose their proliferative activity. But RBCs do not contain a nucleus and thus are not prone to radiation damage.[17,18] However, irradiation treatment requires special large-scale radiation equipment and strict radiation protection management. Not all medical institutions have such conditions, and irradiation treatment cannot usually be completed in the operating room, which directly affects its wide clinical promotion.

A leukoreduction filter is usually a microporous filter composed of multilayer polyester nonwoven fabric or hollow glass fiber, which is usually used to remove large white blood cells (WBCs) by physical barrier (pore size) and charge adsorption to reduce the incidence of a variety of adverse transfusion reactions. The smaller, smooth and seedless RBCs can pass smoothly through the filter. In general, the use of WBC filters to treat whole blood can reduce the number of WBCs by 4 or 5 log.[19] Studies of several scholars have suggested that a leukoreduction filter can also effectively remove tumor cells mixed in the salvaged RBCs, so its application in autologous blood salvage can solve the problem of the pollution of shed tumor cells to salvaged blood.[20–22] Nonetheless, some scholars suspect that capability of leukoreduction filtering tumor cells is load limited, and a leukoreduction filter is able to reduce number of tumor cells in salvaged blood to a great extent, but when tumor cells number to a high order of magnitude and a leukoreduction filter will fail to remove completely tumor cells in the salvaged blood, the risk of distant spread of the tumor remains.[6,23–25]

Irradiation and leukoreduction filtration have achieved relatively satisfactory results in the inactivation or clearance of tumor cells in salvaged blood, but there are still some shortcomings. The inactivation of viruses, bacteria, lymphocytes, and protozoa in whole blood by riboflavin photochemical treatment (RPT) has been applied.[26–29] Based on this, we propose that RPT can be used to inactivate tumor cells in salvaged blood. It is hoped that tumor cells will be inactivated by RPT with minimal damage to RBCs. However, there are some differences between them. On the one hand, the biologic activity of tumor cells is different from that of bacteria, viruses, lymphocytes, and protozoa, and the difference in tolerance to RPT is not known. The inactivation effect of tumor cells cannot be inferred from the effect of inactivating bacteria, viruses, and protozoa, which needs to be confirmed by experimental data. On the other hand, a series of operations, such as negative pressure suction, anticoagulation, filtration, and washing will cause additional damage to RBCs, and it is not known whether salvaged RBCs can tolerate RPT. Therefore, on the basis of our previous inactivation of circulating tumor cells by RPT,[30] the HCT116 tumor cells in simulated salvaged blood were treated with RPT to evaluate the proliferation ability of HCT116 tumor cells and the damage of RBCs and to verify the safety and feasibility of RPT inactivation HCT116 tumor cells in salvaged blood.

MATERIALS AND METHODS

Materials

Riboflavin sodium phosphate injection (5 mL: 10 mg) was purchased from Jiangxi Pharmaceutical Co., Ltd. Transparent blood bags of ethylene-vinyl acetate material with a capacity of 10 mL were purchased from Sichuan Nangel Biotechnology Co., Ltd. Whole blood CD326 (EpCAM) microbeads, whole blood column kit, LS separation columns, autoMACS rinsing solution, MACS BSA stock solution, MidiMACS separator, CD45-FITC, and CD326-PE monoclonal antibodies were purchased from Miltenyi Biotec. An apoptosis detection kit (PE Annexin V Apoptosis Detection Kit I) was purchased from BD Biosciences. A free hemoglobin test kit and an adenosine triphosphate (ATP) assay kit were purchased from Nanjing Jiancheng Bioengineering Institute. Ultraviolet (UV) A lamp tubes (365 nm) were purchased from Philips. UVB lamp tubes (310 nm) were purchased from Sanyo. Phosphate-buffered saline and RPMI 1640 were purchased from HyClone. Trypsin and fetal bovine serum were purchased from Gibco. An automated chemistry analyzer (Combas 8000) was purchased from Roche. An auto-transfusion system (Cell Saver Elite) and supporting consumables were purchased from Haemonetics. HCT116 cells were obtained from the Tumor Laboratory of Medical School of Chinese PLA and passaged every other day, and third-generation cells were selected for use. NOD/SCID mice were purchased from Vital River Laboratory Animal Technology Co., Ltd. Fresh whole blood units were obtained within 2 hours after collection from the Department of Blood Transfusion, the First Medical Center, Chinese PLA General Hospital.

All clinical samples were collected with written informed consent under the approval of the Institutional Review Board at the Chinese PLA General Hospital. All experiments were performed according to the Declaration of Helsinki. All protocols and procedures for experiments using mice were approved by the Ethics Committee for Animal Experimentation of Chinese PLA General Hospital.

RPT of whole blood from healthy donors mixed with HCT116 cells (simulated salvaged blood containing tumor cells)

Ten healthy blood donors were recruited and 400 mL of whole blood treated with anticoagulant citrate dextrose was collected from each donor. Each bag of blood was divided into four equal parts, and a certain number of HCT116 cells were mixed into 100 mL of whole blood to obtain a final concentration of 5×10^5/mL. A riboflavin sodium phosphate injection with a final concentration of 50 μmol/L was added, and after mixing, 100 mL of mixed blood was divided into 20 aliquots, and each 5 mL was transferred to an ethylene vinyl acetate blood storage bag and illuminated in an RPT device we developed. A mixed light source consisting of UVA (365 nm) and UVB (310 nm) was used, and the ambient temperature was controlled at 6 ± 2°C. According to the different illumination doses, the blood samples were assigned to Experimental Groups 1 (illumination dose: 18 J/cm^2), 2 (illumination dose: 23.4 J/cm^2), and 3 (illumination dose: 28.8 J/cm^2), respectively; meanwhile, a riboflavin-free sample without UV illumination served as the control group. Twenty aliquots of blood from the same donor and treated with the same illumination dose was remixed into an empty blood bag for subsequent washing.

Washing the treated mixture with a blood salvage machine (simulated washing to salvaged blood)

The Cell Saver Elite system was installed according to the operating instructions. A salvage line was prefilled with normal saline, and 100 mL of riboflavin photochemical treated blood was aspirated into the salvage line (at a minimum pressure of 50 mm Hg selected for manual suction) after preflush. After the blood was completely inspired, the line was flushed with normal saline. The FILL button was then manually clicked so that the liquid in the salvage line was injected into a selected 125-mL centrifuge cup. When residual RBCs in the salvage line were completely collected, the WASH button was clicked for washing with 750 mL of normal saline (only one cycle). The system automatically discharged the final product into a blood bag. The EMPTY button was clicked to evacuate the residual blood in the salvage line.

Sorting and enrichment of HCT116 cells

After RPT and washing, about 100 mL of a mixture of RBCs and HCT116 tumor cells was added into 250 μL of CD326 beads and incubated at 4°C for 30 minutes, then the whole blood column was loaded onto a magnetic grate, to which a strainer was added, and the strainer and the whole blood column were moistened with 5 mL of matching buffer (autoMACS rinsing solution: MACS BSA stock solution = 19:1). The mixture of incubated beads and RBCs was then added into the whole blood column, flushed with 3 mL of matching buffer three times, then 5 mL of eluent was added to wash cells out under pressure, and the cells were collected in a new 15-mL centrifuge tube. Thereafter, the whole blood column was removed and replaced with a new LS column, which was then rinsed with 5 mL of matching buffer. The cells washed out by pressurized eluent were passed through the LS column, which was washed with 3 mL of matching buffer three times, then 5 mL of matching buffer was used to pressurize the column and wash the cells out again. Next, the cells were centrifuged and resuspended in RPMI 1640 medium.

Incubation of sorted HCT116 cells

Sorted HCT116 cells were incubated in RPMI 1640 containing 5% fetal bovine serum, 100 U/mL of penicillin and 100 mg/mL of streptomycin, while the medium was placed in an 5% CO_2 moistened incubator at 37°C.

Apoptosis assay of HCT116 cells

After the sorted HCT116 cells had been incubated for 24 hours, an apoptosis assay was performed using the PE Annexin V apoptosis assay kit according to instructions.

Tumorigenicity assay in NOD/SCID mice

Twenty-four female NOD/SCID mice aged 28 to 41 days were randomly divided into control group and Experimental Groups 1, 2, and 3, with six mice in each group. In the control group, 100 μL of HCT116 cells (5×10^6 cells) without RPT were injected into the armpit of each mouse's left limb. In Experimental Group 1, 100 μL of HCT116 cells (5×10^6cells) treated with 50 μmol/L of riboflavin and 18 J/cm^2 UV illumination were injected into the armpit of each mouse's left limb. In Experimental Group 2, 100 μL of HCT116 cells (5×10^6cells) treated with 50 μmol/L riboflavin and 23.4 J/cm^2 UV illumination were injected into the armpit of each mouse's left limb. In Experimental Group 3, 100 μL of HCT116 cells (5×10^6 cells) treated with 50 μmol/L riboflavin and 28.8 J/cm^2 UV illumination were injected into the armpit of each mouse's left limb. The mice were euthanized 3 weeks after injection of HCT116 cells, and tumor xenografts were blunt dissected and removed. The length and short diameter of the tumor were measured by calipers, and the tumor size was calculated according to the tumor volume = [length × (width)^2]/2. Tumor xenografts were sliced for hematoxylin and eosin (HE) staining.

Hemolysis assay of RBCs

The samples of the control group and Experimental Groups 1, 2, and 3 after the RPT and washing procedure were centrifuged at 1760 × *g* for 5 minutes, and the supernatant was collected. Then Reagents 1, 2, and 3 were mixed at 20:20:1 in

a micro-free hemoglobin detection kit to form a chromogenic reagent; 0.15 mL of supernatant and 2.5 mL chromogenic reagent were then added to the determination tube, and 0.15 mL of deionized water and 2.5 mL of chromogenic agent were added to the blank control tube. The substances in each tube were mixed well and incubated in a water bath at 37°C for 20 minutes. The optical density (OD) of each tube was determined at 510 nm and then substituted into a calculation formula fitted by the standard curve. After the free hemoglobin was detected, the hemolysis rate was calculated according to the formula reported in the literature.[31]

Assay of biochemical parameters of salvaged blood supernatant

The resuspension blood samples in four groups after the RPT and washing procedure were centrifuged at 1760 × *g* for 5 minutes, and the supernatant was collected and tested for the concentrations of K^+, Na^+, Cl^-, Ca^{2+}, CO_2, and lactate dehydrogenase (LDH) using the Cobas 8000 chemistry analyzer.

ATP assay of RBCs

RBCs (0.5 mL) from each sample were diluted with double-distilled water according to the volume ratio of 1:4, then mixed to make the hemolysis complete, and the hemolytic blood was prepared. The hemolytic blood was heated and boiled in a glass test tube for 10 minutes. The samples were mixed well, and centrifuged at 3000 × *g* for 10 minutes. The supernatant was taken out, and the OD of each tube was measured with a spectrophotometer that was set at a wavelength of 636 nm and a light diameter of 0.5 cm. Double-distilled water was used to adjust zero. All steps were performed according to the kit instructions.

ATP (μmol/gHb) = (measured OD value-control OD value)/(standard OD value-blank OD value) × 10^3 × dilution multiple/hemoglobin concentration

Statistical processing

Data are expressed in the form of mean ± standard deviation and were statistically analyzed by t test in statistical analysis software (SPSS 17.0, IBM) where $p < 0.05$ indicates the presence of statistical differences. Plotting and data analysis were performed using OriginPro 8.5.

RESULTS

Apoptosis of HCT116 cells

The apoptosis rates of HCT116 in the control group and Experimental Groups 1, 2, and 3 were 7.52 ± 0.69%, 52.94 ± 1.12%, 61.46 ± 2.79%, and 66.14 ± 4.57%, respectively. P values in Experimental Group 1 versus control group, Experimental Group 2 versus control group, and Experimental Group 3 versus control group were 2.2E-12, 2.8E-10, and 6.2E-09, respectively (Fig. 1). Compared with the control group, the apoptosis rate in the experimental groups (Groups 1, 2, and 3) increased remarkably with increasing UV illumination dose.

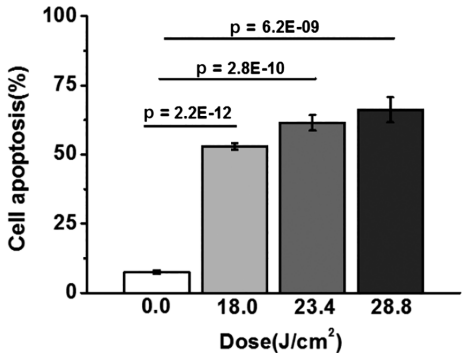

Fig. 1. Comparison of apoptosis rates of HCT116 cells in control group (absence of both riboflavin and UV light) and three experimental groups.

Tumorigenicity test in NOD/SCID mice

All the six mice in the control group developed tumors, and the average tumor volume was 0.608 ± 0.380 cm^3, whereas no tumor growth was found in totally 18 mice in Experimental Groups 1, 2, and 3 (Table 1). The left subaxillary tissue of each group was stripped, sliced, and stained with HE (Fig. 2). The results showed that the tissue block stripped from the control group was HCT116 tumor tissue. The stripped tissues in Experimental Groups 1, 2, and 3 were striated muscle tissue, and no HCT116 tumor cells were found.

TABLE 1. Tumor volume data for mice in various groups

Group	No.	Length (cm)	Width (cm)	Volume (cm^3)
Control group (absence of both riboflavin [RF] and ultraviolet [UV])	1	1.4	1.3	1.183
	2	1.5	1.0	0.750
	3	1.3	1.2	0.936
	4	1.1	0.9	0.446
	5	1.0	0.7	0.163
	6	0.7	0.7	0.172
Experimental Group 1 (RF, 50 μmol/L; UV, 18 J/cm^2)	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
	5	0	0	0
	6	0	0	0
Experimental Group 2 (RF, 50 μmol/L; UV, 23.4 J/cm^2)	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
	5	0	0	0
	6	0	0	0
Experimental Group 3 (RF, 50 μmol/L; UV 28.8 J/cm^2)	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
	5	0	0	0
	6	0	0	0

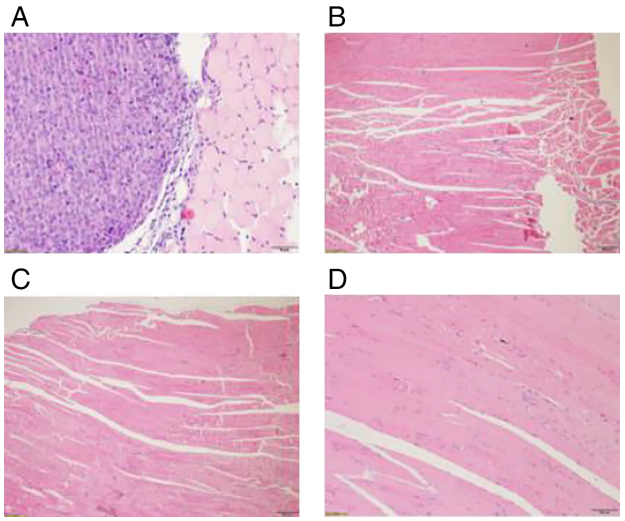

Fig. 2. HE staining results of HCT116 tumor cell sections in various groups. (A) Control group, the tumor cells arranged in nests were seen under the microscope, and were heteromorphic. The mitosis was easy to be seen, and the tumor invaded the surrounding striated muscle tissue; (B) Experimental Group 1, striated muscle tissue under microscope, no tumor cells; (C) Experimental Group 2, striated muscle tissue under microscope, no tumor cells; and (D) Experimental Group 3, striated muscle tissue under microscope, no tumor cells. [Color figure can be viewed at wileyonlinelibrary.com]

Free hemoglobin in supernatants of salvaged RPT-treated RBCs

Free hemoglobin levels of supernatants in the control group and Experimental Groups 1, 2, and 3 were 0.14 ± 0.09 g/L, 0.32 ± 0.09 g/L, 0.36 ± 0.08 g/L, and 0.41 ± 0.07 g/L, respectively. We calculated the p values of the different experimental groups versus the control group, and the results were as follows: p = 0.00081 for Experimental Group 1 versus the control group, p = 0.00007 for Experimental Group 2 versus the control group, and p = 0.000002 for Experimental Group 3 versus the control group (Fig. 3).

Hemolysis rates of salvaged riboflavin photochemical-treated RBCs

The hemolysis rates in the control group and Experimental Groups 1, 2, and 3 were $0.07 \pm 0.04\%$, $0.15 \pm 0.04\%$, $0.17 \pm 0.04\%$, and $0.20 \pm 0.03\%$, respectively (Fig. 4). We compared the differences in hemolysis rates between the control group and the experimental groups, and the results are as follows: p = 0.00081 for Experimental Group 1 versus the control group, p = 0.00007 for Experimental Group 2 versus the control group, and p = 0.000002 for Experimental Group 3 versus the control group. RPT on salvaged blood led to increased hemolysis rates of RBCs ($p < 0.05$), but such hemolysis rates were much less than the quality control criterion for hemolysis rate of whole blood and blood components at the end of the preservation period.

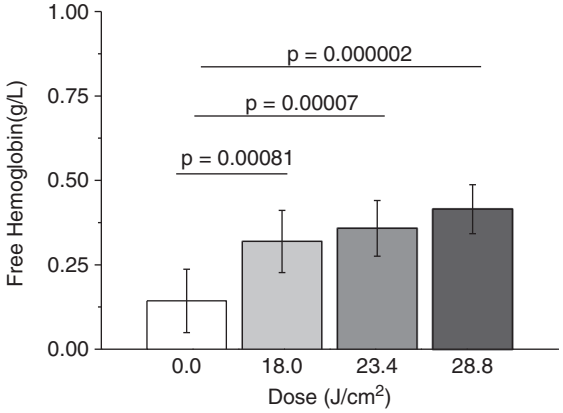

Fig. 3. Free hemoglobin levels in various groups.

Measurements of biochemical parameters of salvaged riboflavin photochemical-treated RBC supernatants

Figure 5 showed that RPT had no evident influence on concentrations of Ca^{2+}, Cl^{-}, CO_2, and Na^{+} in supernatants of salvaged blood ($p > 0.05$). RPT damaged the salvaged RBCs to some extent, as K^{+} concentration in Experimental Groups 2 and 3 was significantly higher than that in the control group ($p < 0.05$). LDH concentration in the experimental groups increased with increasing illumination dose, without statistical difference as compared with that in the control group ($p > 0.05$).

ATP levels of salvaged riboflavin photochemical-treated RBCs

ATP levels in the control group and Experimental Groups 1, 2, and 3 were 2.78 ± 1.99 μmol/gHb, 2.64 ± 1.40 μmol/gHb, 2.89 ± 2.11 μmol/gHb, and 3.54 ± 1.95 μmol/gHb, respectively (Fig. 6). We compared the differences in ATP levels between

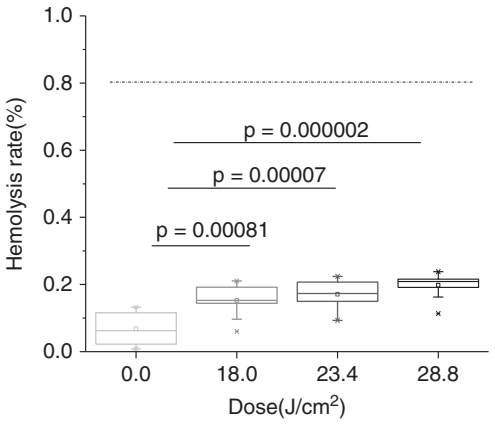

Fig. 4. Hemolysis rates of simulated salvaged blood in various groups.

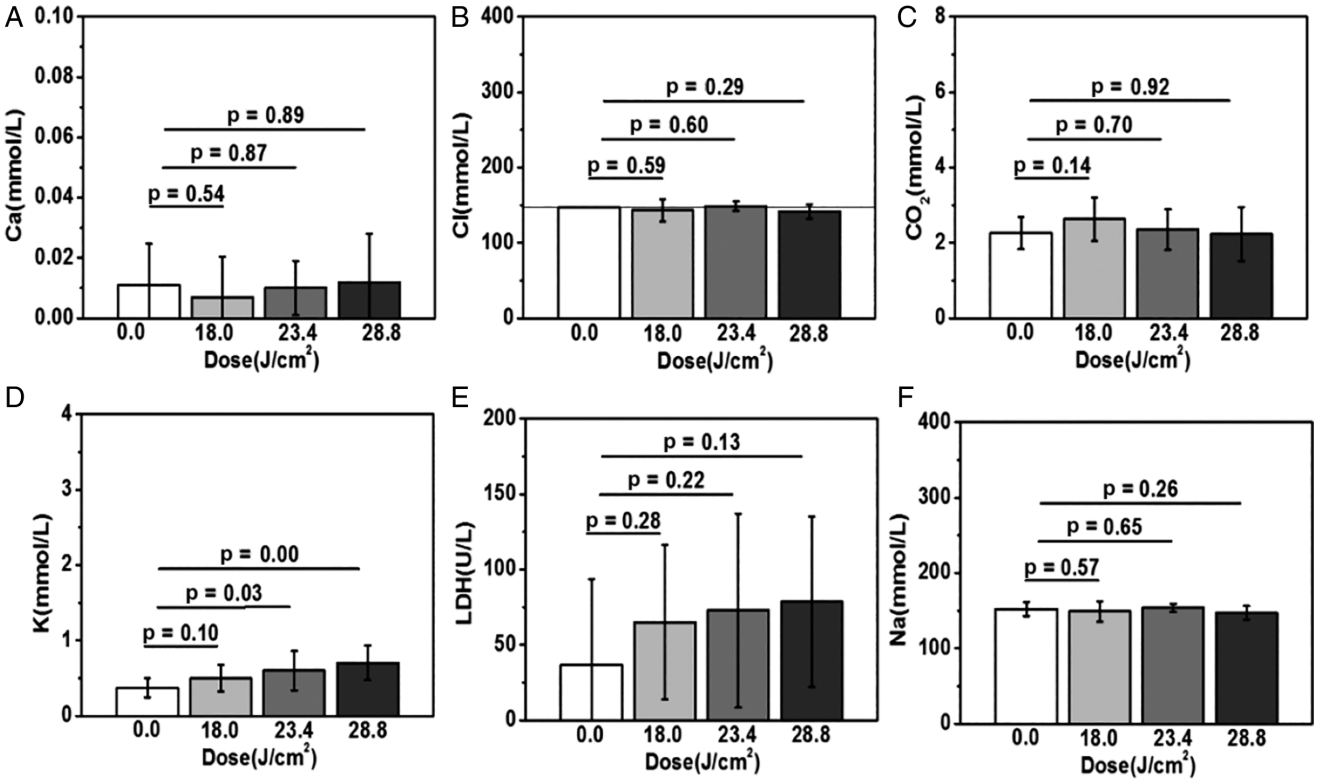

Fig. 5. Comparative statistical histogram of primary biochemical parameters in various groups. (A) Concentration of calcium ion in various groups; (B) Concentration of chloride ion in various groups; (C) Concentration of carbon dioxide in various groups; (D) Concentration of potassium ion in various groups; (E) Concentration of lactate dehydrogenase in various groups; and (F) Concentration of sodium ion in various groups.

the experimental groups and the control group. The results are as follows: p = 0.87 for Experimental Group 1 versus the control group, p = 0.91 for Experimental Group 2 versus the control group, and p = 0.43 for Experimental Group 3 versus the control group. Based on the results, RPT had no significant impact on ATP level of salvaged riboflavin photochemical-treated RBCs ($p > 0.05$).

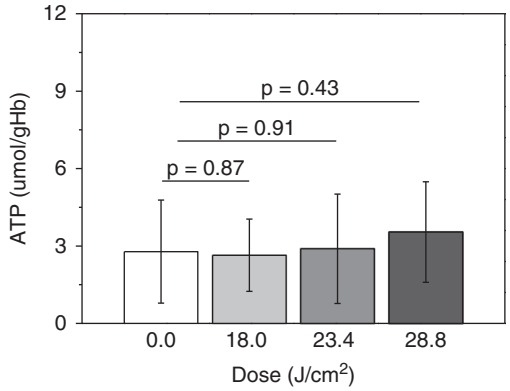

Fig. 6. ATP levels of salvaged riboflavin photochemical-treated RBCs in various groups.

DISCUSSION

IBS in malignant solid tumor surgery has been regarded as a contraindication for many years. The main concern is the contamination of salvaged RBC components by malignant tumor cells falling off during the operation, resulting in the distant dissemination of malignant tumor cells through blood flow. Previous studies have confirmed that proliferative tumor cells can indeed be detected in salvaged RBCs from patients with malignant tumors and that the return of these tumor cells to patients may lead to tumor dissemination.[32,33] Hansen and colleagues[13] found that a 50-Gray irradiation dose can inactivate tumor cells mixed in salvaged blood. However, the high price and unmovable characteristics of blood irradiation equipment limit its wide application. The tumor cell load in the shed blood ranges from 10 to 10^7 cells,[6] and only a 4 to 5 log reduction in tumor cell number will be obtained after filtration.[13] However, tumor metastasis can originate from the expansion of a single tumor cell.[34] The two methods mentioned above have been tried to apply in inactivation and clearance of tumor cells from salvaged blood, which make it possible to use IBS for tumor surgery. However, there are still some limitations in these two methods, which limit their wide application.

The results of our previous study showed that RPT under certain conditions could inactivate proliferative HCT116 cells in peripheral blood, and at the same time, coagulation, immune function, and RBC damage are controlled within acceptable limits.[30] RPT is expected to be an auxiliary means to eliminate perioperative hematogenous metastasis of malignant tumors. Therefore, it is also feasible to use RPT technology to deal with the blood salvaged from patients with malignant tumors during operation and inactivate the tumor cells. Different from the normal anticoagulant whole blood extracted from the vein, the blood salvaged from the operative field experienced a continuous nonphysiologic process including primary coagulation activation, negative vacuum suction, filtration, washing, and resuspension, and RBCs have suffered a certain degree of mechanical damage.[2] It remains unclear whether salvaged RBCs can tolerate the superimposed damage caused by RPT. Therefore, we studied the inactivation of HCT116 tumor cells and the injury of RBCs to establish the technical conditions that can be used to salvage blood of patients with tumors during the operation, so that it can promote the wide application of IBS in patients with tumors.

Immunodeficient mice lack the ability to reject foreign tissue, so they are widely used in the study of xenotransplantation of various tumor models.[35,36] Our study showed that RPT can induce apoptosis of most HCT116 cells in Experimental Groups 1, 2, and 3 (see Fig. 1), and this damage prevents them from forming tumors in NOD/SCID immunodeficient mice. In the control group (without RPT), the tumor cells after sorting and enrichment could be tumorigenic in NOD/SCID immunodeficient mice (Table 1 and Fig. 2). The results showed that HCT116 tumor cells after salvage and washing had the ability to proliferate and may still lead to distant metastasis of the tumor by direct reinfusion, and RPT under certain conditions can inactivate the HCT116 cells mixed in the salvaged blood, so that they lose the ability to proliferate.

In this study, the superimposed damage to RBCs caused by the whole process including RPT and the blood salvage procedure (negative vacuum suction, filtration, washing, etc.) was systematically evaluated. The results showed that RPT on salvaged blood could lead to the increase of free hemoglobin in the supernatant and hemolysis rate of salvaged RBCs (Figs. 3 and 4), but such results were much lower than the quality control criteria for RBC components at the end of the preservation period that the hemolysis rate should not be more than 0.8% and free hemoglobin should not be higher than 2 g/L.[37,38] The concentrations of Na^+, Cl^-, Ca^{2+}, and CO_2 in supernatant of Experimental Groups 1, 2, and 3 were not significantly different from those of the control group ($p > 0.05$). Compared with the control group, there was no significant difference in K^+ concentration in Experimental Group 1 ($p > 0.05$), and the concentration of K^+ in Experimental Groups 2 and 3 significantly increased ($p < 0.05$), but lower than that in normal human blood. The concentration of LDH increased with the increase of illumination dose, but there was no significant difference compared with the control group ($p > 0.05$) (Fig. 5). Therefore, RPT can cause some damage to salvaged RBCs, but it should not bring additional burden and damage to the body after reinfusion.

ATP is the main energy substance in RBCs and plays an important role in maintaining the normal operation of the cell membrane sodium-potassium ion pump, the constant concentration of calcium ion in RBCs, the continuous renewal of lipid components in the membrane of RBCs, and normal morphology, function, in vivo survival time of RBCs, and so on. ATP is often used as one of the important indexes to evaluate the quality of RBCs.[39] In this study, the effect of RPT on the change of ATP content in salvaged RBCs was evaluated. The results showed (Fig. 6) that compared with the control group, there was no significant difference in the content of ATP in salvaged RBCs among the experimental groups ($p > 0.05$), which indicated that RPT had limited damage to the function of salvaged RBCs during operation.

CONCLUSION

RPT (50 μmol/L riboflavin and 18 J/cm^2 UV illumination radiation) can effectively inactivate HCT116 tumor cells mixed in salvaged blood and make them lose their ability to proliferate, and the damage to the structure and function of RBCs is relatively minimal. The main quality indexes of salvaged RBCs were within the standard range. Therefore, RPT technology is expected to make the application of IBS in patients with malignant solid tumors more safe and convenient.

STUDY LIMITATIONS

As colorectal cancer surgery usually brings contamination, the IBS technique will not be used in general. However, due to the limitation of tumor cell sorting technology, the yield of HCT116 cells separated by CD326 whole blood sorting magnetic beads is the highest, which can meet the requirements of the number of cells in subsequent tumorigenesis experiments. We tried to use CD326 whole blood sorting magnetic beads to separate Hep G2 cells and HeLa cells from simulated salvaged RBCs and separate MCF-7 cells using Nectin-4, and the isolation rates of these three tumor cell lines were too low to enrich enough tumor cells for follow-up experiments. Therefore, we had to choose HCT116 cells for the study of RPT on salvaged RBCs. In this study, whole blood of healthy donors was used to simulate intraoperative salvaged blood and was anticoagulated with anticoagulant citrate dextrose, solution A instead of heparin. Compared with the blood salvaged in the surgical field, whole blood of healthy donors enabled more timely anticoagulation with fewer blood clots. Variations in

hematocrit or hemolysis of incoming blood, anticoagulant used, and blood bag storage material can affect the extent of RBC damage.

CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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