

Effect of conditioned medium from low-dose X-ray irradiated cells on doxorubicin-induced leukemic K562 cells death

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ABSTRACT

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Background: This current determined the effects of conditioned medium from low-dose X-ray irradiated cells on cytotoxicity of doxorubicin (Dox) in leukemic K562 cancer cells. **Materials and Methods:** Cells were X-irradiated with 0, 0.02, 0.05, and 0.1 Gy, and then these cells were cultured for 24 h. The culture medium collected from irradiated cells was transferred to non-irradiated cells followed by treatments with 50 and 100 nM of Dox. The biological endpoints, cell viability, lipid peroxidation, intracellular reactive oxygen species (ROS) and intracellular iron, were determined at 48 h after treatment. **Results:** The results showed that irradiated cells conditioned medium (ICCM) alone significantly decreased in cell viability when compared with control cells. The combination of ICCMs with Dox did not change the cell viability when compared with corresponding controls while it had significant decreases in cell viability when compared with treated cells of 50 and 100 nM Dox alone, respectively. The cell viability effects were used to calculate the synergism quotient values, resulting in values that showed non-synergistic effects in all combination conditions. ICCMs and combination of ICCMs with Dox significantly decreased in lipid peroxidation levels but did not change the intracellular ROS and the intracellular iron levels in K562 cells. **Conclusion:** These findings revealed that the ICCMs could induce cell death in K562 cells and ICCMs were contributed to the cytotoxicity of Dox in K562 cells. It should be noted that the lipid peroxidation might have involved to ICCMs-induced cell death.

INTRODUCTION

It is well known that high doses of radiation are deleterious to directly irradiated cells or tissue ⁽¹⁻⁴⁾. However, several results from studies on the effects of radiation using dissimilar biological responses such as DNA breaks, chromosomal aberrations, micronucleus and cell death have shown that there are no deleterious effects due to direct exposure to low doses of radiation ⁽⁵⁻¹¹⁾.

However, there is much evidence that ionizing radiation induces changes not only in the directly irradiated cells but also in the non-irradiated neighboring cells. This radiation response is known as radiation induced non-targeted effects which includes bystander effects, as well. For radiation induced bystander effects, it has been suggested that the irradiated cells somehow communicate to the non-irradiated neighboring cells. The studies shows that this communication involves transmitted signals sent through gap junctions and soluble factors (i.e.; cytokines and growth factors) that are secreted into the culture medium ⁽¹²⁻¹⁹⁾. There are reports that directly irradiated cells or irradiated cells conditioned medium co-cultured with non-irradiated

cells results in various biological responses in non-irradiated cells such as DNA breaks, chromosomal aberrations, and cell death in ways similar to directly irradiated cells ^(20, 21).

Previously, we had studied the effects of low-dose radiation after an *in-vitro* direct exposure of normal human blood cells (i.e.; lymphocytes and red blood cells) and cancerous human blood cells (i.e.; leukemic K562 and multidrug-resistant leukemic K562/*adr* cancer cells). These results suggested that there were no harmful effects from the low-dose radiation when normal human blood cells were directly exposed in an *in-vitro* condition ⁽²²⁻²⁵⁾. In contrast, low-dose radiation increased reactive oxygen species (ROS) and impaired mitochondria in directly exposed K562 and K562/*adr* cancer cells ⁽²⁵⁾.

Leukemia is a commonly diagnosed malignant tumor of the blood cells. It has a high mortality rate and accounts for 3.1% of cancer deaths worldwide ^(26, 27). Treatment for leukemia depends on the types of leukemia. Current treatments are chemotherapy, radiotherapy, immunotherapy, and bone marrow transplantation. Of note, chemotherapy is the basis for all treatment regimens ⁽²⁸⁻³⁰⁾. The anthracycline drugs like doxorubicin and its derivatives are used as

chemotherapeutic agents in the treatment of leukemia⁽³¹⁾.

Based on our previous results, we found that ROS increases in directly irradiated leukemic K562 cells that were exposed to low-dose radiation⁽²⁵⁾. In addition, one of these anticancer drugs actions was believed to be involved in ROS generation⁽³¹⁻³³⁾. Due to the fact that radiation response could be found not only in the directly irradiated cells but also in the non-irradiated neighboring cells which was a radiation-induced bystander effect, an interesting question could then be considered. "Do irradiated cells conditioned medium modify the cytotoxicity of doxorubicin in cancer cells?" The aim of this current study was to determine the effects of conditioned medium obtained from low-dose X-ray irradiated cells on doxorubicin-induced leukemic K562 cell death. This study will provide the data on effect of irradiated cells conditioned medium on the cytotoxicity of doxorubicin in cancer cells. Nonetheless, these data will help improve understanding of the fundamental radiation biology of low dose X-rays.

MATERIALS AND METHODS

Experimental design

The experimental design is shown in figure 1. Briefly, the K562 cancer cells were irradiated to X-rays at total radiation doses of 0, 0.02, 0.05, and 0.1 Gy. The medium was collected at 24 h post-irradiation, defined as an irradiated cell conditioned medium (ICCM). The ICCM was transferred to non-irradiated cells. Next, these cells were treated with 0, 50, and 100 nM Dox. The biological endpoints; cell morphology, cell viability, synergism quotient (SQ) calculation, intracellular ROS, lipid peroxidation, and intracellular iron were determined at 48 h after treatments with ICCMs, Dox, and combination of ICCMs and Dox.

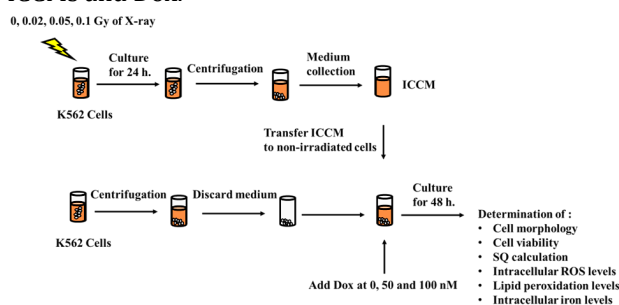


Figure 1. The experimental design. ICCM; irradiated cells conditioned medium, Dox; doxorubicin, SQ; synergism quotient, ROS; reactive oxygen species.

Chemicals

Resazurin, 2',7'-dichlorofluorescein diacetate (DCHF-DA), 2-thiobarbituric acid (TBA), Potassium thiocyanate, Doxorubicin and Acridine orange (AO) were obtained from Sigma-Aldrich (St Louis, MO,

USA). Fetal bovine serum (FBS) and Penicillin/streptomycin (Pen/Strep) were purchased from Capricorn Scientific. RPMI-1640 medium was obtained from Caisson Labs. Sodium hydroxide (NaOH, RCI labscan, Thailand). Propidium iodide (PI).

Irradiation

A medical diagnostic X-ray machine manufactured by Shimadzu Corporation, Japan, was utilized in the present study. The machine was operated with a tube potential of 100 kilovoltage peak (kVp) and a tube current of 200 milliamperere (mA). The parameters recorded from this X-ray system are presented in table 1.

Table 1. The parameters were recorded from the medical X-ray machine operating at 100 kVp, 200 mA, and a range of exposure times.

Setting			Measurement			
Tube Potential (kVp)	Tube current (mA)	Exposure time (Sec)	Dose rate (Gy.min ⁻¹)	HVL (mmAl)	Target / filter	Total dose (Gy)
100	200	0.14	9.1	4.7	W/AI	0.02
100	200	0.36	9.1	4.7	W/AI	0.05
100	200	0.71	9.1	4.7	W/AI	0.1

Cell line and cell culture

The human leukemia cell line used in this study was K562, obtained from the RIKEN BRC CELL BANK in Japan. The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin, maintained in a humidified incubator with 5% CO₂ at 37% °C. The initial cell density was 1 × 10⁵ cells. mL⁻¹, which increased to approximately 8-10 × 10⁵ cells. mL⁻¹ after 72 h. Routine sub-culturing was performed every 72 h⁽³⁴⁾.

For the experiment, the initial cell density was 5×10⁵ cells. mL⁻¹, and after 24 h, it increased to 8-10×10⁵ cells. mL⁻¹, indicating that the cells had entered the exponential growth phase.

ICCM collection

The K562 cancer cells were irradiated by X-rays at total radiation doses of 0.02, 0.05, and 0.1 Gy. Non-irradiated cell served as the control group. Later, irradiated and non-irradiated cells were grown in a humidified atmosphere with 5% CO₂ at 37°C for 24 h. These cells were centrifuged and then were collected the medium. This collected medium was defined as ICCM.

Morphological observation of viability and death cells

The AO and PI morphological assessment of viability and death cells was carried out as described in previous reports^(35,36). A double-staining dye was freshly prepared by using an AO/PI mixture of (AO,10 mg. mL⁻¹; PI, 10 mg mL⁻¹, 1:1; v/v). The dye was kept in an ice bath in dark conditions. The initial number of cells was at 2×10⁶ cell. mL⁻¹ were then observed in

terms of cell morphology at 48 h after treatment. Briefly, the cell suspension was incubated with the dye mixture for 5 min and then were transferred onto a glass slide for fluorescence viewing. The slides were coded, yet there was no awareness regarding the treatment until after the slides were completely analyzed. Slides were then observed under a fluorescence microscope within 30 min.

Cell viability assay

The cell viability assay was performed by using the resazurin assay described in previous reports ^(37, 38) with minor modifications. The experiment began with an initial cell concentration of 5×10^4 cells. mL⁻¹, and cell viability was assessed 48 h post-treatment. Viability was measured by adding 100 μ L of resazurin solution (0.1 mg. mL⁻¹) to the cell cultures. After a 4-h incubation period, absorbance was measured at 570 and 600 nm using a UV-Vis spectrophotometer (Shimadzu Corporation, Japan). The %cell viability was calculated by equation (1):

$$\frac{(\text{Abs. at 570 nm} - \text{Abs. at 600 nm}) \text{ treated cells}}{(\text{Abs. at 570 nm} - \text{Abs. at 600 nm}) \text{ control}} \times 100 \quad (1)$$

SQ calculation

The SQ value was calculated by dividing the net cell viability effect of the combination of ICCM and Dox by the sum of individual cell viability effect of ICCM and Dox. The SQ value higher than 1.0 indicated a synergistic effect.

Determination of intracellular ROS levels

Intracellular ROS levels were measured using the DCHF-DA assay, as previously described in earlier studies ⁽³⁹⁾ with minor modification. The initial number of cells was at 2×10^5 cell. mL⁻¹ and intracellular ROS was determined at 48 h after treatments. Briefly, cells were suspended in 2 mL HEPES Na⁺-buffer, pH 7.25 at 37 °C and were vigorously stirred with a cuvette. Next, 100 nM DCHF-DA was added to the system. The DCHF-DA was hydrolyzed by intracellular esterase to non-fluorescent 2',7'-dichlorofluorescein (DCHF). The DCHF reacted with intracellular ROS to form fluorescent 2',7'-dichlorofluorescein (DCF). The DCF fluorescence intensity at 523 nm (excite at 502 nm) was recorded as a function of time using a luminescence spectrometer (Perkin Elmer, USA) as indicated in figure 2. The dF/dt value was calculated at 100 sec after adding DCHF-DA to the system. This dF/dt value referred to the slope value which reflected intracellular ROS.

Determination of intracellular iron

Intracellular iron levels were measured using the iron-thiocyanate assay. Cells were initially seeded at a density of 2×10^6 cells. mL⁻¹, and measurements were taken 48 h after treatment. In summary, cells were lysed with an acid mixture of hydrochloric acid

and nitric acid (1:1 v/v) at 60 °C for 2 h. Potassium thiocyanate was then added to the lysate. The absorbance at 480 nm, corresponding to intracellular iron content, was measured using a UV-Vis spectrophotometer (Shimadzu Corporation, Japan).

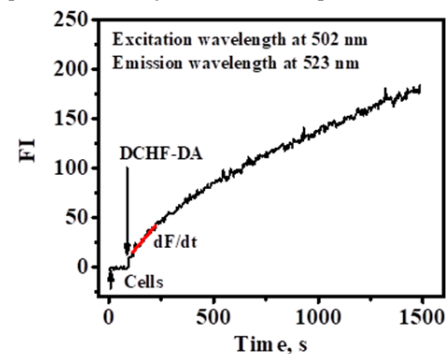


Figure 2. Typical of DCHF-DA uptake into the cells. The fluorescence intensity at $\lambda_{em} = 523$ nm ($\lambda_{ex} = 502$ nm) was recorded as a function of time. The dF/dt value referred slope value which reflexed to intracellular ROS. FI; fluorescence intensity, DCHF-DA; 2',7'-dichlorofluorescein diacetate, and s; Second.

Determination of lipid peroxidation levels

Lipid peroxidation was assessed using the thiobarbituric acid reactive substances (TBARS) assay. The initial cell density was adjusted to 2×10^6 cells. mL⁻¹, and measurements were taken 48 h post-treatment. In brief, the cells were incubated with a 2 mg. mL⁻¹ TBA solution at 80 °C for 1 h. The TBA solution was prepared in a 1:1 (v/v) mixture of 50 mM NaOH and glacial acetic acid. After incubation, the mixture was cooled to room temperature, and absorbance at 530 nm, indicative of lipid peroxidation was measured using a UV-Vis spectrophotometer (Shimadzu Corporation, Japan).

Statistical analysis

Data are presented as the mean $\pm$ standard error of the mean (SEM). Statistical differences between the test groups and the control group were analyzed using an independent Student's t-test. A p-value below 0.05 was considered statistically significant.

RESULTS

Morphological observation

The AO/PI staining images displayed viability and death cells in the treated cells and non-treated cells in terms of color as indicated in figure 3. The green color and orange color cells were referred to as viability and death cells, respectively. The green color displayed a high number of cells in ICCM at 0 Gy while ICCMs at 0.02, 0.05 and 0.1 Gy were found to have a high number of orange color cells.

The combination of ICCMs at 0.02, 0.05, and 0.1 Gy with 50 nM Dox increased the amount of orange color cells when compared to treated cell with 50 nM Dox alone. Similar results were obtained using a combination of ICCMs at 0.02, 0.05 and 0.1 Gy with

100 nM Dox also having increases in orange color cells when compared to treated cell with 100 nM Dox alone.

These results suggested that ICCMs alone and combination of ICCMs with Dox could induce cell death at 48 h after treatment.

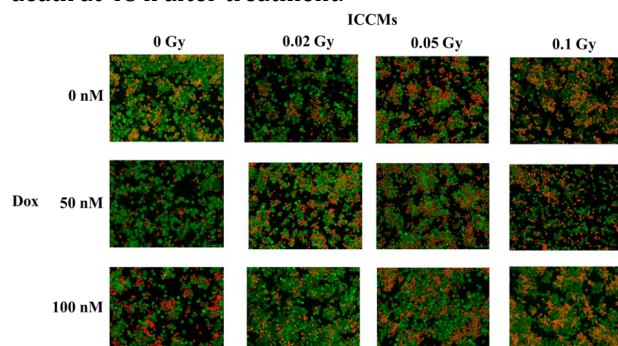


Figure 3. Images of AO/PI fluorescence images stained K562 cells treated with ICCMs, Dox and combination of ICCMs and Dox at 48 h. green color and orange color were defined as viability and death cells, respectively. Magnifications 100X. AO; acridine orange, PI; propidium iodide, ICCM; irradiated cells conditioned medium, and Dox; doxorubicin.

Cell viability

Figure 4A shows the effect of ICCMs, Dox, and combination of ICCMs and Dox on cell viability of K562 cancer cells at 48 h. The results suggest that ICCMs at 0.02, 0.05, and 0.1 Gy alone significantly decreased cell viability when compared with ICCM at 0 Gy.

The data demonstrated that 50 and 100 nM Dox significantly decreased the viability of K562 cancer cells to 62 and 59% at 48 h, respectively. The combination of ICCMs at 0.02, 0.05 and 0.1 Gy with 50 nM Dox did not change the cell viability when compared with ICCMs at 0.02, 0.05, and 0.1 Gy alone, while there were significant decreases in cell viability when compared with treated cells with 50 nM Dox alone. Similar results were obtained by the combination of ICCMs at 0.02, 0.05, and 0.1 Gy with 100 nM Dox not changing the cell viability when compared with ICCMs at 0.02, 0.05, and 0.1 Gy alone. Again, there were significant decreases in cell viability when compared with treated cells with 100 nM Dox alone.

The SQ values calculation

As revealed in table 2, the SQ values were less than 1 for all combination conditions. The SQ value calculation of cell viability indicated a non-synergistic effect of the combination of ICCMs and Dox in K562 cells.

Intracellular ROS levels

Figure 4B shows the effect of ICCMs, Dox, and combination of ICCMs and Dox on intracellular ROS in K562 cancer cells at 48 h. This data shows no change in the dF/dt values in cells treated with

ICCMs at 0.02, 0.05, and 0.1 Gy alone when compared with non-treated cells (ICCM at 0 Gy).

Table 2. SQ values calculation for K562 cancer cells viability.

Combinations	SQ values
ICCM 0.02 Gy / Dox 50 nM	0.29
ICCM 0.05 Gy / Dox 50 nM	0.33
ICCM 0.1 Gy / Dox 50 nM	0.35
ICCM 0.02 Gy / Dox 100 nM	0.31
ICCM 0.05 Gy / Dox 100 nM	0.34
ICCM 0.1 Gy / Dox 100 nM	0.35

SQ; synergism quotient, ICCM; irradiated cell conditioned medium, Dox; doxorubicin.

Similar results were obtained from the combination of ICCMs at 0.02, 0.05, and 0.1 Gy with 50 and 100 nM Dox not changing the dF/dt values when compared with ICCMs at 0.02, 0.05, and 0.1 Gy alone or 50 and 100 Dox alone, respectively.

These results suggested that ICCMs alone and combination of ICCMs with Dox did not change the intracellular ROS levels in K562 cancer cells at 48 h.

Intracellular iron

Figure 4C shows the effect of ICCMs, Dox and combination of ICCMs and Dox on intracellular iron in K562 cells at 48 h. This data shows no change in the Abs. at 480 nm values in cells treated with ICCMs at 0.02, 0.05, and 0.1 Gy alone when compared with non-treated cells (ICCM at 0 Gy).

More similar results were obtained from the combination of ICCMs at 0.02, 0.05, and 0.1 Gy with 50 and 100 nM Dox not changing the Abs. at 480 nm values when compared with ICCMs at 0.02, 0.05, and 0.1 Gy alone or 50 and 100 Dox alone.

These results suggested that ICCMs alone and combination of ICCMs with Dox did not change the intracellular iron in K562 cancer cells at 48 h.

Lipid peroxidation level

Figure 4D shows the effect of ICCMs, Dox and combination of ICCMs and Dox on lipid peroxidation level in K562 cancer cells at 48 h. The results suggested that ICCMs at 0.02 and 0.05 Gy alone significantly decreased in Abs. at 530 nm, except for ICCM at 0.1 Gy alone when compared with ICCM at 0 Gy.

The combination of ICCMs at 0.02, 0.05, and 0.1 Gy with 50 nM Dox significantly decreased in Abs. at 530 nm when compared to treated cells with 50 nM Dox alone. Similar results were obtained from the combination of ICCMs at 0.02, 0.05, and 0.1 Gy with 100 nM Dox having significantly decreased in Abs. at 530 nm when compared to treated cells with 100 nM Dox alone.

These results suggested that ICCMs alone and combination of ICCMs with Dox significantly decreased lipid peroxidation level in K562 cancer cells at 48 h.

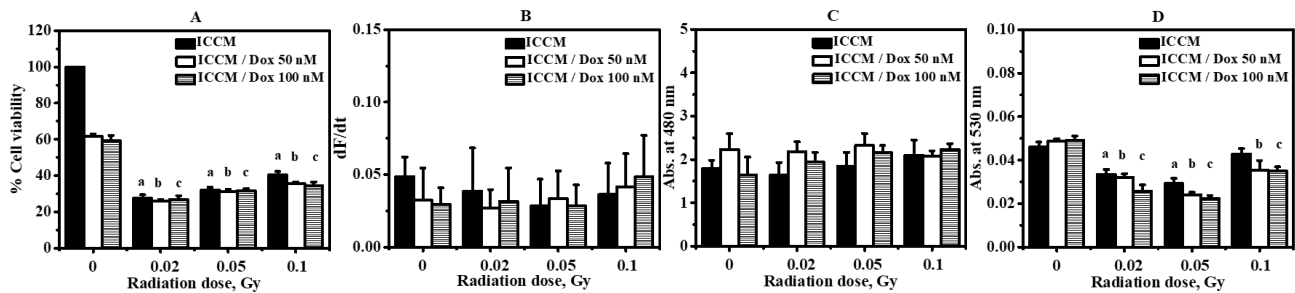


Figure 4. Effect of ICCMs at 0.02, 0.05 and 0.1 Gy, Dox and combination of ICCMs and Dox on K562 cancer cells at 48 h. (A) cell viability, (B) dF/dt values were referred to as intracellular ROS. (C) Abs. at 480 nm values were referred to as intracellular iron and (D) Abs. at 530 nm values were referred to as lipid peroxidation level. a, b and c P<0.05 when compared with ICCM at 0 Gy, Dox 50 nM and Dox 100 nM alone, respectively. ICCM; irradiated cells conditioned medium, Dox; Doxorubicin, and Abs.; Absorbance.

DISCUSSION

There was a previous study that determined that effect of low-dose radiation on K562 cancer cells after direct exposure in *in vitro*. The results showed that direct exposure to low-dose radiation induced ROS incrementally and that there was mitochondrial damage in K562 cancer cells (25). The results in this current study were similarly detected in previous studies regarding the cell viability conducted by Bahreyni Toossi *et al.* (20) and Faqihi *et al.* (21). These authors have used cell viability to study the effects of ICCM from human lung tumor QU-DB cells (20) and human glioblastoma U87MG cells (21). Their results indicated that ICCMs significantly decreased cell viability in non-irradiated QU-DB cells and U87MG cells. However, it should be noted that there was a difference in the experimental design between this study and the studies conducted by Bahreyni Toossi *et al.* (20) and Faqihi *et al.* (21) regarding the cell types and radiation dosages used to investigate the effects of ICCM. This current study used human leukemic K562 cells and radiation doses ranging from 0.02-0.1 Gy, while human lung tumor QU-DB cells and radiation doses ranging from 0.5-8 Gy were used in the study conducted by Bahreyni Toossi *et al.* (20) and human glioblastoma U87MG cells with radiation dosages set at 4 Gy were used in the study conducted by Faqihi *et al.* (21). Herok *et al.* (40) showed that increased frequencies of micronucleated and apoptotic cells occurred after growth in ICCM at 4 Gy of X-rays. The authors also showed that ICCM did not induce cell cycle arrest in K562 cells.

Furthermore, this current study investigated the possible mechanisms of ICCMs-induced cell death. ICCMs-induced K562 cancer cells death might not involve intracellular ROS and intracellular iron. Of note, the pattern of intracellular ROS changes did correspond to changes in lipid peroxidation for ICCMs-treated cells. This was not surprising because lipid peroxidation can be caused from an oxidative attack on the unsaturated acyl chains of lipids by ROS (41, 42). These current findings were consistent with a study conducted by Sokolov and Neumann (43). These authors assessed the effect of ICCMs at 0.2, 2, and 10 Gy of X-rays on human bone-marrow mesenchymal

stem cells and embryonic stem cells. The authors suggested that ICCMs showed no evidence of apoptotic cells and/or DNA damage induction compared to control cells. In contrast, Soleymanifard *et al.* (44, 45) investigated the micronucleus induction in human lung tumor QU-DB cells and normal human lung fibroblast MRC5 cells after being treated by the ICCMs at 0.5, 1, 2, and 4 Gy of gamma rays from radioactive ^{60}Co . The authors suggested that the number of cells containing micronucleus in ICCM-treated QU-DB and MRC5 cells was significantly higher than that of the control group, while the ICCMs from human breast cancer MCF-7 cells at 2, 4, 6, 8, and 10 Gy of X-rays induced angiogenic responses in endothelial cells (46). Hence, it should be noted that ICCMs-induced beneficial or harmful effects on non-irradiated cells depending on a variety factor such as cell type, radiation dose, radiation type, and biological endpoints.

For combination effect, it might be proposed that ICCM can enhance the cytotoxic effects of Dox in K562 cancer cells. However, combinations of ICCMs and Dox decreased lipid peroxidation levels in K562 cancer cells at 48 h, compared with Dox alone but did not for ICCMs alone. These effects on lipid peroxidation seem to oppose the cell viability effects. This might be due to a time-dependence effect. Therefore, time-dependence factors should be studied in the future.

CONCLUSION

In conclusion, these findings suggested that the ICCMs at 0.02, 0.05, and 0.1 Gy of X-rays could induce cell death in K562 cancer cells. ICCMs were contributed to the cytotoxicity of Dox in K562 cells. It should be noted that the lipid peroxidation might have involved to ICCMs-induced cell death.

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Conflict of interest: The authors declare that they have no conflict of interests.

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Ethical approval: None.

Author contributions statement: SW, contributed to experimental design, experimental work, and results analysis. SK, contributed to experimental work, results analysis, resources and draft writing. MT, contributed to experimental design, experimental work, results analysis, resources, draft writing and final writing. All authors read the manuscript and approved the final version.

AI usage: None.

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