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Title	Ionizing radiation induces mitochondrial reactive oxygen species production accompanied by upregulation of mitochondrial electron transport chain function and mitochondrial content under control of the cell cycle checkpoint
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1 **Title**

2 Ionizing radiation induces mitochondrial ROS production
3 accompanied by upregulation of mitochondrial electron
4 transport chain function and mitochondrial content under
5 control of the cell cycle checkpoint

6

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22

1 **Abstract**

2 While ionizing radiation (Ir) instantaneously causes the
3 formation of water radiolysis products that contain some
4 reactive oxygen species (ROS), ROS are also suggested to be
5 released from biological sources in irradiated cells. It is now
6 becoming clear that these secondarily generated ROS after Ir
7 have a variety of biological roles. Although mitochondria are
8 assumed to be responsible for this Ir-induced ROS production,
9 it remains to be elucidated how Ir triggers it. Therefore, we
10 conducted this study to decipher the mechanism of Ir-induced
11 mitochondrial ROS production. In human lung carcinoma A549
12 cells, Ir (10 Gy of X-rays) induced a time-dependent increase
13 of the mitochondrial ROS level. Ir also increased mitochondrial
14 membrane potential, mitochondrial respiration, and
15 mitochondrial ATP production, suggesting upregulation of the
16 mitochondrial electron transport chain (ETC) function after Ir.
17 Although we found that Ir slightly enhanced mitochondrial ETC
18 complex II activity, the complex II inhibitor 3-nitropropionic
19 acid failed to reduce Ir-induced mitochondrial ROS production.
20 Meanwhile, we observed that the mitochondrial mass and
21 mitochondrial DNA level were upregulated after Ir, indicating
22 that Ir increased mitochondrial content of the cell. Because
23 irradiated cells are known to undergo cell cycle arrest under
24 control of the checkpoint mechanisms, we examined the

1 relationships between the cell cycle and mitochondrial content
2 and cellular oxidative stress level. We found that the cells
3 in the G2/M phase had a higher mitochondrial content and
4 cellular oxidative stress level than cells in the G1 or S phase,
5 regardless of whether the cells were irradiated. We also found
6 that Ir-induced accumulation of the cells in the G2/M phase led
7 to an increase of cells with a high mitochondrial content and
8 cellular oxidative stress level. This suggested that Ir
9 upregulated mitochondrial ETC function and mitochondrial
10 content, thereby resulting in mitochondrial ROS production and
11 that Ir-induced G2/M arrest contributed to the increase of the
12 mitochondrial ROS level by accumulating cells in the G2/M phase.

13

14 Keywords: ionizing radiation, mitochondrial ROS, electron
15 transport chain, cell cycle

16

1 **Introduction**

2 Ionizing radiation (Ir) initially causes ionization and
3 excitation of water, leading to the formation of water
4 radiolysis products such as hydrated electron (e_{aq}^-), ionized
5 water (H_2O^+), hydroperoxyl radical (HO_2^\bullet), hydroxyl radical
6 ($^\bullet OH$), hydrogen radical (H^\bullet), hydrogen peroxide (H_2O_2) in a very
7 short period of time ($\sim 10^{-8}$ sec) when irradiated to a biological
8 system [1]. Except H_2O_2 , they are unstable and disappear within
9 less than 10^{-3} sec [1]. A significant part of the initial damage
10 done to cells by Ir is due to DNA damage, and these water
11 radiolysis products (especially $^\bullet OH$) play an important role in
12 this process [2, 3]. Some of water radiolysis products such as
13 $^\bullet OH$ and HO_2^\bullet are also called reactive oxygen species (ROS), a
14 group of chemically reactive molecules containing oxygen. ROS
15 have been suggested to be involved in a variety of biological
16 processes ranging from cell proliferation to carcinogenesis.
17 The main ROS that have marked biological effects are superoxide
18 radical ($O_2^{\bullet -}$), H_2O_2 , $^\bullet OH$, peroxy radical (ROO^\bullet), alkyl
19 hydroperoxide ($ROOH$) and singlet oxygen (1O_2) [4]. Most ROS are
20 labile and dissipate quickly except H_2O_2 and $ROOH$, which are
21 relatively stable. When irradiated to cells, not only Ir leads
22 to the generation of ROS derived from water radiolysis, it has
23 been shown that Ir increases the intracellular level of ROS
24 including $O_2^{\bullet -}$ several hours after its exposure [5, 6],

1 indicating that Ir also stimulates ROS production derived from
2 biological sources. It is becoming clear that the secondarily
3 generated ROS after Ir have a variety of biological roles. These
4 include apoptotic signaling, genomic instability after Ir, and
5 radiation-induced bystander effects [7-12]. These effects
6 ultimately have an impact on cellular integrity and survival.
7 Therefore, it is of considerable significance to determine the
8 mechanism underlying the Ir-induced cellular ROS production
9 from the aspect of radiation biology.

10 Thus far, there is conflicting evidence on the source of
11 the secondarily generated ROS after Ir. Though it is reported
12 that NADPH oxidase is involved in Ir-induced ROS production [5,
13 13], several studies, including ours, suggested that
14 mitochondria are responsible for it [7, 14, 15]. However, it
15 remains unclear how Ir induces ROS production from mitochondria.
16 In general, it has been postulated that electron leakage from
17 mitochondrial electron transport chain (ETC) complexes to
18 molecular oxygen causes the generation of $O_2^{\bullet-}$ and its derivative
19 ROS (such as H_2O_2 and $\bullet OH$) in mitochondria (hereafter
20 "mitochondrial ROS") [16-18]. Mitochondrial respiration for
21 ATP production leads to mitochondrial ROS production, and
22 0.12-2% of O_2 incorporated for respiration is estimated to go
23 to $O_2^{\bullet-}$ under *in vitro* conditions [16]. ETC complex inhibitors
24 such as rotenone and antimycin A potentiate mitochondrial $O_2^{\bullet-}$

1 production by enhancing this electron leakage, indicating that
2 the electron flow through ETC strongly influences mitochondrial
3 ROS production [16, 19].

4 Although it has been suggested that Ir promotes
5 mitochondrial ROS production, it remains to be elucidated how
6 Ir triggers it. Therefore, we conducted this study to decipher
7 the mechanism of Ir-induced mitochondrial ROS production.

8

9 **Materials and Methods**

10 *Reagents*

11 2',7'-Dichlorofluorescein diacetate (DCFDA) and
12 3-nitropropionic acid (3-NP) were obtained from Sigma-Aldrich
13 (St. Louis, MO). Tetramethylrhodamine methyl ester (TMRM) and
14 MitoSOX Red (MSR) were purchased from Invitrogen (Carlsbad, CA).
15 ATP assay kits were from TOYO B-Net Co. (Tokyo, Japan).
16 Nuclear-ID Red DNA stain was obtained from Enzo Life Sciences
17 (Farmingdale, NY). Rotenone, carbonyl cyanide m-chlorophenyl
18 hydrazine (CCCP), oligomycin and other reagents were obtained
19 from Wako Pure Chemical Co. (Osaka, Japan).

20

21 *Cell culture and treatment*

22 Human lung carcinoma A549 cells were maintained in RPMI1640
23 medium (Invitrogen) supplemented with 10% fetal bovine serum
24 (RPMI1640/10% FBS) at 37°C in 5% CO₂. Human cervical carcinoma

1 HeLa S3 cells were grown as suspension cultures in Joklik's-MEM
2 (Sigma-Aldrich) containing 10% FBS at 37°C in 5% CO₂.
3 X-irradiation was performed with an X-ray generator (Shimadzu
4 HF-350; Kyoto, Japan) and the dose rate was 3.9 Gy/min at 200
5 kVp, 20 mA with a 1.0 mm aluminum filter, which was determined
6 using Fricke's chemical dosimeter. Drugs were added immediately
7 after irradiation, with incubation for the indicated times.

8

9 *Cellular oxidative stress levels and mitochondrial ROS* 10 *production*

11 The fluorescent probe DCFDA was used for the assessment of
12 cellular oxidative stress levels [20, 21]. Cells were incubated
13 with RPMI1640/10% FBS containing 10 μM DCFDA for 30 min at 37°C.
14 Then they were trypsinized and washed twice with PBS(-). After
15 resuspending the cells in serum-free RPMI1640 medium, they were
16 analyzed using an EPICS XL flow cytometer (Beckman Coulter,
17 Fullerton, CA). The mean DCFDA fluorescent intensity of each
18 sample was normalized to that of a control sample to calculate
19 the relative DCFDA intensity.

20 Another fluorescent probe MSR was used for the assessment
21 of mitochondria-derived O₂^{•-} and other oxidants (such as H₂O₂
22 and [•]OH) [20, 22, 23]. Cells were incubated with RPMI1640/10%
23 FBS containing 2 μM MSR for 30 min at 37°C. Then they were
24 trypsinized and washed twice with PBS(-). After resuspending

1 them in serum-free RPMI1640 medium, the cells were analyzed as
2 described above.

3

4 *Mitochondrial membrane potential*

5 The fluorescent probe TMRM was used for the assessment of
6 mitochondrial membrane potential [22]. Cells were incubated in
7 RPMI1640/10% FBS containing 20 nM TMRM for 30 min at 37°C. Then
8 they were trypsinized and washed twice with PBS(-). After
9 resuspending the cells in serum-free RPMI1640 medium, they were
10 analyzed using an EPICS XL flow cytometer. The mean TMRM
11 fluorescent intensity of each sample was normalized to that of
12 a control sample to calculate the relative TMRM intensity.

13

14 *Oxygen consumption analysis by polarography*

15 Cells were trypsinized and washed twice with PBS(-). Then they
16 (5×10^6 cells) were resuspended in buffer E (0.3 M mannitol,
17 10 mM KCl, 5 mM MgCl₂, 1 mg/ml BSA, 10 mM KH₂PO₄ [pH 7.4]) and
18 transferred to a sample chamber. Oxygen consumption by the cells
19 was monitored using a YSI 5300 biological oxygen monitor (YSI
20 Life Sciences, Yellow Springs, OH). Rotenone (final conc. = 2
21 μM) was added to the cell suspension with a microsyringe.

22

23 *Measurement of oxygen consumption rate by electron spin*
24 *resonance (ESR) spectroscopy*

1 The peak-to-peak line width of the ESR spectrum of lithium
2 5,9,14,18,23,27,32,36-octa-*n*-butoxy-2,3-naphthalocyanine
3 (LiNc-BuO) shows a linear response to the partial pressure of
4 oxygen (pO₂) and has been used extensively to measure oxygen
5 consumption *in vitro* [24, 25]. LiNc-BuO was synthesized
6 according to the method described previously [26, 27]. At
7 indicated periods after 10 Gy of X-irradiation, cells were
8 collected and washed. The cells (5 × 10⁵ cells) were suspended
9 in 100 μl of serum-free medium containing 0.2 mg LiNc-Buo and
10 2% dextran to avoid sedimentation of the cells and LiNc-BuO
11 particles. The cell suspension was immediately drawn into a
12 glass capillary tube, which was then sealed at both ends. The
13 ESR measurements were carried out using a JEOL-RE X-band
14 spectrometer (JEOL, Tokyo, Japan) with a cylindrical TE011 mode
15 cavity (JEOL). The cavity was maintained at 37°C using a
16 temperature controller (ES-DVT4, JEOL). Spectrometer
17 conditions were as follows: incident microwave power, 10 mW;
18 modulation frequency, 100 kHz; field modulation amplitude,
19 63 μT; and scan range, 0.5 mT. The spectral line widths were
20 analyzed using a Win-Rad Radical Analyzer System (Radical
21 Research, Tokyo, Japan) and converted into pO₂ values according
22 to the following equation described by Fujii *et al.* [27].

23

24 pO_2 (mmHg) = (LW × 10-340)/10.33 (LW; ESR line width [μT])

1

2 To examine the relationship between mitochondrial
3 respiration and cellular oxygen consumption, rotenone was added
4 at 2 μ M where indicated.

5

6 *Cellular ATP content*

7 Cellular ATP content was evaluated with the ATP assay kit
8 according to the provider's protocol. In brief, cells were
9 collected and resuspended in RPMI1640/10% FBS. The cells (5 x
10 10^3 cells/100 μ l) were transferred to wells of 96-well plate,
11 followed by the addition of ATP assay reagent (100 μ l). After
12 incubating the plate for 30 min at 25°C, chemiluminescence from
13 each well was measured with a luminometer (Luminescencer-JNR;
14 ATTO, Tokyo, Japan) set at 25°C.

15

16 *Isolation of mitochondria*

17 Intact mitochondria were isolated from HeLa S3 cells. At 12 h
18 after X-irradiation, the cells were collected and resuspended
19 in ice-cold isolation buffer (2.5 mM HEPES-KOH [pH 7.4], 70 mM
20 sucrose, 220 mM mannitol, 1 mM EGTA). A nitrogen cell disruption
21 vessel, model 4639 from Parr Instrument Company (Moline, IL),
22 was used to burst cells. The cell suspension was placed into
23 the cell disruption chamber and put under a pressure of 250 psi
24 for 30 min at 4°C. After unbroken cells and nuclei were removed

1 by centrifuging the suspension at 1000 g for 10 min at 4°C, the
2 supernatant was further centrifuged at 10,000 g for 10 min at
3 4°C to precipitate mitochondria. The final mitochondrial pellet
4 was resuspended in isolation buffer and the protein
5 concentration was determined using the Bio-Rad protein assay
6 (Bio-Rad, Hercules, CA).

7

8 *Mitochondrial ETC enzyme activities*

9 Complex I activity was measured as the rotenone (2
10 µg/ml)-inhibitable rate of NADH (0.325 mM) oxidation in 25 mM
11 KH₂PO₄ (pH 7.2), 5 mM MgCl₂, 2.5 mg/ml BSA, 65 µM Coenzyme Q₁
12 (Sigma-Aldrich), 2 µg/ml antimycin A, and 2 mM KCN. The rate
13 of the absorbance change at 340 nm with a 425 nm reference
14 wavelength was measured. To measure complex II activity,
15 mitochondrial extract was incubated in a mixture of 25 mM KH₂PO₄
16 (pH 7.2), 20 mM sodium succinate, 5 mM MgCl₂, 150 µM
17 2,6-dichlorophenolindophenol (DCPIP), 2 mM KCN, 2 µg/ml
18 antimycin A, and 10 µg/ml rotenone. Sixty-five µM Coenzyme Q₁
19 was then added to the mixture and the rate of DCPIP reduction
20 was monitored at 600 nm with a 750 nm reference wavelength.
21 Complex IV activity was measured by the rate of oxidation of
22 cytochrome c (II) (15 µM) in 25 mM KH₂PO₄ (pH 7.2) and 0.45 mM
23 n-dodecyl β-maltoside. The rate of the absorbance change at 550
24 nm with a 580 nm reference wavelength was measured. Complex

1 I+III activity was measured as the rotenone (2
2 µg/ml)-inhibitable rate of reduction of cytochrome c (III) (80
3 µM) in 50 mM KH₂PO₄ (pH 7.2), 0.2 mM NADH, and 2 mM KCN. The
4 rate of the absorbance change at 550 nm with a 580 nm reference
5 wavelength was read. For complex II+III activity, mitochondrial
6 extract was incubated in a mixture of 40 mM KH₂PO₄ (pH 7.2),
7 20 mM sodium succinate, 0.5 mM EDTA, 2 mM KCN, and 10 µg/ml
8 rotenone. Cytochrome c (III) (62.5 µM) was then added to the
9 mixture and its reduction rate was monitored at 550 nm with a
10 580 nm reference wavelength. All mitochondrial ETC enzyme
11 activities were normalized to the total protein amount.

12

13 *Mitochondrial mass*

14 Mitochondrial mass was measured by staining cells with a
15 membrane potential-independent mitochondrial dye MitoTracker
16 Green FM (Invitrogen) [22]. Cells were incubated in
17 RPMI1640/10% FBS containing 50 nM MitoTracker Green FM for 30
18 min at 37°C. Then they were trypsinized and washed twice with
19 PBS(-). After being resuspended in PBS(-), the cells were
20 analyzed using an EPICS XL flow cytometer.

21

22 *Mitochondrial DNA content*

23 Mitochondrial DNA (mtDNA) content was determined as a marker
24 for mitochondrial density using quantitative RT-PCR as

1 previously reported [28, 29]. In brief, total DNA was isolated
2 from the cells using a FastPure DNA kit (TAKARA BIO, Shiga,
3 Japan) according to the manufacturer's instructions. To
4 evaluate the mtDNA content, the relative amounts of D-loop and
5 cytochrome c oxidase subunit II (COXII) (mtDNA-encoded) were
6 determined by the $\Delta\Delta\text{Ct}$ method using β 2-microglobulin (β 2M)
7 (nuclear DNA-encoded) as an internal control.

8

9 *Simultaneous flow cytometric analysis of cell*
10 *cycle/mitochondrial content and cell cycle/cellular oxidative*
11 *stress levels using a double-staining technique*

12 For simultaneous analysis of the cell cycle and mitochondrial
13 content, we used two fluorescent probes, Nuclear-ID Red and
14 MitoTracker Green for staining the nuclear DNA and
15 mitochondrial membrane, respectively. After irradiated cells
16 were trypsinized and washed with PBS(-), they were incubated
17 with serum-free RPMI1640 containing 20 μM Nuclear-ID Red and
18 40 nM MitoTracker Green for 30 min at 37°C. The cells were then
19 analyzed using an EPICS XL flow cytometer. MitoTracker Green
20 fluorescence of the cell fraction corresponding to the G1, S,
21 and G2/M phases was analyzed to determine the mitochondrial
22 content of the cells in each cell cycle phase. The mean
23 MitoTracker Green fluorescence intensity of each sample was
24 normalized to that of a control sample to calculate relative

1 MitoTracker Green intensity. For simultaneous analysis of the
2 cell cycle and cellular oxidative stress levels, we applied the
3 method described above using 10 μM DCFDA as a probe for the
4 cellular oxidative stress instead of MitoTracker Green.

5

6 *Statistical analysis*

7 All results were expressed as means \pm SE of at least three
8 separate experiments. Statistical analyses were performed with
9 Student's *t*-test. The minimum level of significance was set at
10 $p < 0.05$.

11

12 **Results**

13 *Ionizing radiation induces mitochondrial ROS production*

14 We first examined the cellular oxidative stress levels in human
15 lung carcinoma A549 cells after Ir using the fluorescent probe
16 DCFDA and a flow cytometer [20, 21]. When A549 cells were
17 incubated for 12 h after X-irradiation (10 Gy), they became more
18 oxidized than the cells collected immediately after irradiation
19 (Fig. 1A). As shown in Fig. 1B, DCFDA fluorescence intensity
20 in A549 cells gradually increased after Ir, peaked at 12 h, and
21 declined at 24 h, consistent with our previous report [7].

22 To test if Ir stimulated mitochondrial ROS production,
23 we analyzed it using the fluorescent probe MSR and a flow
24 cytometer [20, 22, 23]. Cells collected 12 h after X-irradiation

1 exhibited higher MSR fluorescence intensity than the cells
2 collected immediately after irradiation (Fig. 1C). As shown in
3 Fig. 1D, Ir increased MSR fluorescence intensity in a
4 time-dependent manner. In addition, we examined mitochondrial
5 ROS production after Ir in various cell lines (HeLa, MKN45, MeWo,
6 NIH3T3) and found that Ir facilitated it in all cell lines tested
7 (data not shown). These results indicated that Ir induced
8 cellular oxidative stress and that mitochondrial ROS were, at
9 least in part, responsible for it.

10

11 *Ionizing radiation increases mitochondrial membrane potential*

12 We next evaluated the mitochondrial membrane potential after
13 Ir using the fluorescent probe TMRM and a flow cytometer [22].
14 When A549 cells were incubated for 12 h after Ir, they showed
15 higher TMRM fluorescence intensity than the cells collected
16 immediately after irradiation (Fig. 2A). As shown in Fig. 2B,
17 the TMRM intensity in A549 cells was time-dependently increased
18 up to 48 h after Ir. To confirm that this change was caused by
19 the increase of mitochondrial membrane potential *per se*, we
20 tested the effect of an uncoupling agent CCCP on TMRM
21 fluorescence intensity. As shown in Fig. 2C, the TMRM intensity
22 was increased after Ir exposure and it was dissipated by the
23 CCCP treatment. Therefore, these results demonstrated that the
24 mitochondrial membrane potential was increased after Ir.

1

2 *Ionizing radiation promotes mitochondrial respiration*

3 We next investigated if Ir affected cellular respiration.

4 Cellular oxygen consumption was monitored using a standard

5 polarographic technique with a Clark-type oxygen electrode. As

6 shown in Fig. 3A, the oxygen concentration in the sample chamber

7 stayed the same in the absence of cells (buffer only). When cells

8 were loaded in the chamber, the oxygen concentration decreased

9 at a constant rate. It was completely inhibited by the addition

10 of rotenone, indicating that the reduction in the oxygen

11 concentration was attributable to mitochondrial respiration.

12 When A549 cells were X-irradiated, they showed higher

13 respiratory activity than the cells collected immediately after

14 irradiation, with a 1.6-fold increase (Figs. 3A and 3B).

15 To support this observation, we further analyzed the

16 cellular oxygen consumption rate by ESR spectrometry using

17 LiNc-BuO as an oxygen sensing probe. The peak-to-peak line width

18 of the ESR spectrum of LiNc-BuO particles broadens as the pO_2

19 increases [26]. When non-irradiated A549 cells were mixed with

20 LiNc-BuO particles and the ESR measurement was performed, the

21 ESR spectra were sharpened in a time-dependent manner,

22 indicating the cellular oxygen consumption (Fig. 4A; left). The

23 sharpening of the spectra was observed more rapidly in

24 X-irradiated cells (Fig 4A; right), suggesting that Ir

1 stimulated cellular oxygen consumption. After the line width
2 of the spectrum was converted into a pO_2 value as described in
3 *Materials and Methods*, the pO_2 was plotted as a function of time
4 (Fig. 4B) and the cellular oxygen consumption rate was
5 calculated (Fig. 4C). Figures 4B and 4C demonstrate that Ir
6 elevated cellular oxygen consumption up to 12 h after
7 irradiation, followed by a decrease at 24 h. These results were
8 consistent with the data obtained by polarography (Figs. 3A and
9 3B). Furthermore, we examined cellular oxygen consumption after
10 Ir in various cell lines (HeLa, MKN45, MeWo, NIH3T3), and found
11 that Ir facilitated it in all cell lines tested (data not shown).

12 We then evaluated the effect of rotenone on the Ir-induced
13 cellular oxygen consumption to determine whether mitochondrial
14 respiration was involved in it. As shown in Figs. 4D and 4E,
15 Ir stimulated cellular oxygen consumption and rotenone
16 suppressed not only basal cellular oxygen consumption but also
17 Ir-stimulated cellular oxygen consumption. These results
18 indicated that mitochondrial respiration was responsible for
19 the basal and Ir-induced cellular oxygen consumption.

20

21 *Ionizing radiation promotes ATP production*

22 Because higher mitochondrial respiration has been linked to the
23 higher cellular energy production, we hypothesized that the
24 upregulation of mitochondrial respiration by Ir led to the

1 upregulation of ATP production. To test this hypothesis, we
2 measured cellular ATP content using a commercially available
3 assay kit. As shown in Fig. 5A, Ir resulted in a time-dependent
4 increase of cellular ATP content up to 24 h after irradiation.
5 When the mitochondrial ATP synthase (F_0/F_1 -ATPase) inhibitor
6 oligomycin was applied to the cells after Ir, it reduced the
7 cellular ATP content stimulated by Ir in a
8 concentration-dependent manner without affecting the basal
9 cellular ATP level (Fig. 5B). These data suggested that Ir
10 facilitated mitochondrial energy production activity.

11

12 *ETC enzyme activities are unaffected by ionizing radiation and*
13 *are not associated with the mitochondrial ROS production*

14 These data prompted us to examine if any of the mitochondrial
15 ETC enzyme activities were affected by Ir. To this end,
16 mitochondria were isolated from irradiated and non-irradiated
17 HeLa S3 cells and ETC enzyme activities were analyzed. While
18 the activities of complex I+III, complex IV, and complex I were
19 almost same with or without Ir, the activities of complex II+III
20 and complex II were slightly increased after Ir although they
21 are not statistically significant (Table 1). This result
22 implied that, at the mitochondrial level, only complex II
23 activity was upregulated by Ir among all ETC enzymes evaluated.

24 To investigate if the complex II activity elevated by Ir

1 was involved in the Ir-induced mitochondrial ROS production,
2 we tested the effect of the complex II inhibitor 3-NP in A549
3 cells. As shown in Fig. 6, Ir promoted mitochondrial ROS
4 production measured by MSR fluorescence, and we did not observe
5 any substantial effect of 3-NP treatment on it. These results
6 suggested that the upregulation of complex II activity by Ir
7 was not associated with the Ir-induced mitochondrial ROS
8 production.

9

10 *Ir-induced cell cycle regulation affects mitochondrial content* 11 *and intracellular ROS level*

12 Because the intracellular ROS level is reported to be affected
13 by mitochondrial content of the cell [30], we then examined
14 whether Ir affected mitochondrial content in cells. We
15 evaluated mitochondrial content using two methods,
16 mitochondrial mass measured by MitoTracker Green staining [22]
17 and mtDNA content measured by quantitative PCR. It was
18 demonstrated that Ir increased the mitochondrial mass (Fig. 7A)
19 as well as mtDNA content (Fig. 7B) in A549 cells, indicating
20 that Ir increased the mitochondrial content of the cell.

21 Recent studies have shown that mitochondrial content can
22 be influenced by the cell cycle of the host cell [31, 32]. Because
23 tumor cells are known to accumulate in the G2 phase of the cell
24 cycle after Ir exposure [33], we further investigated whether

1 the cell cycle arrest triggered by Ir was involved in the
2 Ir-induced upregulation of the mitochondrial content and
3 cellular oxidative stress. A technique for double-staining of
4 live cells with Nuclear-ID Red and MitoTracker Green for
5 staining nuclear DNA and mitochondrial membrane, respectively,
6 enabled us to analyze the mitochondrial content of the cell
7 fraction in specific phases of the cell cycle. Figure 8A shows
8 typical flow cytometric profiles in A549 cells after Ir analyzed
9 by Nuclear-ID Red fluorescence from double-stained cells. It
10 was demonstrated that the number of cells in the G2/M phase
11 time-dependently increased up to 12 h after Ir. MitoTracker
12 Green fluorescence of the cell fraction corresponding to the
13 G1, S, and G2/M phases was analyzed to determine the
14 mitochondrial content of the cells in each phase of the cell
15 cycle. The flow cytometric profiles of MitoTracker Green
16 fluorescence from each cell cycle fraction at the indicated
17 times after Ir are overlaid and shown in Fig. 8B, which
18 demonstrates that the cell fraction in the G2/M phase had the
19 highest mitochondrial content, followed by those in the S phase
20 and G1 phase. Moreover, it also shows that Ir-induced
21 accumulation of the cells in the G2/M phase resulted in an
22 increase of cells with a high mitochondrial content. This was
23 confirmed by the graph in Fig. 8C, which demonstrates the
24 relative mitochondrial content during the cell cycle after Ir.

1 The mitochondrial content was increased in the order G1, S, G2/M
2 regardless of the time after irradiation.

3 We then analyzed the cellular oxidative stress levels
4 during the cell cycle by applying the same method using DCFDA
5 as a probe for the cellular oxidative stress instead of
6 MitoTracker Green. We could not use MSR in this system because
7 it has excitation/emission characteristics similar to
8 Nuclear-ID Red. Figures 9A and 9B demonstrate that, similar to
9 the results in Fig. 8: 1) Ir induced cell cycle arrest in the
10 G2/M phase; 2) the cell fraction in the G2/M phase had the highest
11 cellular oxidative stress level, followed by those in the S
12 phase and G1 phase; 3) consequently, Ir-induced accumulation
13 of the cells in the G2/M fraction resulted in an increase of
14 the cells with a high cellular oxidative stress level. Figure
15 9C shows that the cellular oxidative stress level was increased
16 in the order G1, S, G2/M regardless of the time after
17 irradiation.

18 Taken together, these data showed that, regardless of
19 whether cells were irradiated, the cells in the G2/M phase had
20 more mitochondria and a higher cellular oxidative stress level
21 than those in the G1 or S phase. Because Ir caused accumulation
22 of cells in the G2/M phase, it led to the increase of the cells
23 with a high mitochondrial content and a high cellular oxidative
24 stress level.

1

2 **Discussion**

3 The aim of the present study was to determine how Ir triggers
4 mitochondrial ROS production. Our results demonstrated that Ir
5 increased mitochondrial membrane potential (Fig. 2),
6 mitochondrial respiration (Figs. 3 and 4), and mitochondrial
7 ATP production (Fig. 5). These data suggested that Ir
8 upregulated mitochondrial ETC function, consistent with
9 reports showing similar data [34, 35]. Meanwhile, ETC enzyme
10 activities were unchanged after Ir except for complex II (Table
11 1). This finding was inconsistent with reports showing that Ir
12 caused no change [36] or reduced [37] ETC enzyme activities.
13 The difference of the assay system utilized by those groups and
14 us might explain this discrepancy. However, further studies
15 will be necessary to determine the effect of Ir on ETC enzymes.
16 In the present study, mitochondrial ROS production was
17 accompanied by increased mitochondrial membrane potential,
18 enhanced mitochondrial respiration, and maintained ETC enzyme
19 activities. This suggested that mitochondrial ROS were released
20 from functionally active mitochondria. While it is often
21 considered that impairment of ETC function leads to
22 mitochondrial ROS production [38], Kadenbach and other groups
23 have reported that mitochondrial $O_2^{\bullet -}$ production increases
24 exponentially as mitochondrial membrane potential increases,

1 especially when it exceeds roughly 140 mV [39-42]. This level
2 of mitochondrial hyperpolarization can be achieved by sustained
3 mitochondrial respiration in the absence of ADP (state 4
4 respiration) [39]. Hence, it is possible that Ir elicited state
5 4 respiration and mitochondrial hyperpolarization, resulting
6 in mitochondrial ROS production. However, the increased
7 mitochondrial ATP production that we observed in this study has
8 not been linked to state 4 respiration. Thus further
9 investigation is required to fully understand the relationship
10 between mitochondrial ETC function and ROS production after Ir.

11 Moreover, we found that Ir increased mitochondrial
12 content (Fig. 7). Nugent *et al.* demonstrated that Ir increased
13 mitochondrial mass [43], and other groups observed increased
14 mtDNA after Ir [37, 44]. These reports are in line with the
15 results shown in the present study. There are two possible
16 mechanisms to explain these events. One is cell cycle-dependent
17 oscillations of mitochondrial content of the cell as will be
18 discussed later, and the other is the upregulation of
19 mitochondrial biogenesis. The increase of mtDNA content after
20 Ir (Fig. 7B) indicates the upregulation of mitochondrial
21 biogenesis. However, the increase of mtDNA content occurred
22 later than that of mitochondrial mass (Fig. 7A). Therefore, it
23 suggests that Ir-induced mitochondrial biogenesis could be an
24 event that takes place independently of the change of

1 mitochondrial mass, or a secondary event caused by the change
2 of mitochondrial mass.

3 In the present study, we observed the cell
4 cycle-dependent change of mitochondrial content (Fig. 8). Other
5 groups also have demonstrated that mitochondrial content of the
6 cell peaks in the G2/M phase during the cell cycle [31, 45, 46].
7 Furthermore, these studies also reported that the increase of
8 the intracellular ROS level is accompanied by an increase of
9 mitochondrial content during the cell cycle, assisting our
10 results. Therefore, it is likely that this cell cycle-dependent
11 oscillation of the mitochondrial mass and cellular oxidative
12 stress level is a common feature of mammalian cells.

13 It is noteworthy that Ir-induced G2/M arrest led to a
14 sustained increase of cells with elevated mitochondrial content
15 and a higher cellular oxidative stress level, presumably
16 thereby resulting in an increase of the oxidative stress level
17 in the whole population of the cells after irradiation. To the
18 best of our knowledge, this is the first report suggesting that
19 Ir-induced cell cycle arrest is involved in the increase of the
20 cellular/mitochondrial ROS level after irradiation. Under this
21 scenario, cells under G2/M arrest are expected to have higher
22 mitochondrial membrane potential, mitochondrial respiration
23 and cellular ATP level than non-irradiated cells. In fact, cell
24 cycle-dependent oscillation of mitochondrial membrane

1 potential and its elevation in the G2/M phase have been reported
2 [31, 46]. We also observed that cells in the G2/M phase had higher
3 mitochondrial membrane potential, which was sustained after Ir
4 (Yamamori T *et al.*, unpublished data). Recently, Kobashigawa
5 *et al.* elegantly demonstrated that Ir-induced mitochondrial
6 fission was essential for mitochondrial ROS production after
7 Ir [47]. Furthermore, Mitra and colleagues reported that
8 mitochondrial fission was increased in the G2/M phase and cells
9 arrested in the G2/M phase by nocodazole exhibited the greatest
10 mitochondrial fission [32]. Taking into account these findings,
11 it might be possible that Ir-induced G2/M arrest leads to
12 mitochondrial fission, followed by the consequential
13 production of mitochondrial ROS.

14 In addition, we would like to emphasize here that
15 Ir-induced cell cycle regulation also affect the oxidative
16 stress that cells receive. As demonstrated in Fig. 9, cells in
17 the G2/M phase have a higher cellular oxidative stress level
18 than cells in other cell cycle phases. Whereas the G2/M phase
19 lasts 5 h in non-irradiated HeLa cells, it becomes about 3 times
20 longer after their exposure to Ir [48, 49]. Because Ir-induced
21 G2/M arrest increases not only the length of the G2/M phase but
22 also the number of cells in the G2/M phase, this implies that
23 irradiated cells undergo substantial oxidative stress due to
24 Ir-induced cell cycle arrest. Because it is widely accepted that

1 oxidative stress plays an important role in biological
2 responses after Ir, Ir-induced G2/M arrest could participate
3 in them via the elevation of oxidative stress.

4 In conclusion, we hypothesize the mechanism of Ir-induced
5 mitochondrial ROS production to be as follows: in proliferating
6 cells, the mitochondrial content, mitochondrial ETC function
7 and mitochondrial ROS level oscillate in a cell cycle-dependent
8 manner, and all parameters peak in the G2/M phase during the
9 cell cycle; cells exposed to Ir accumulate in the G2/M phase
10 under the control of the G2/M checkpoint, resulting in a
11 corresponding increase of the mitochondrial content and
12 mitochondrial ROS level. Although we assume that this mechanism
13 is primarily responsible for Ir-induced mitochondrial ROS
14 production, it is possible that concomitant events induced by
15 Ir such as mitochondrial fission and/or a change in the ETC
16 function play a role in mitochondrial ROS production in concert
17 with the cell cycle-regulated mechanism. Thus further
18 investigation is required to determine whether Ir-induced
19 mitochondrial ROS production can be explained solely by the cell
20 cycle-regulated mechanism, or other factors are involved in it.

21

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6 Life Science Foundation (T.Y. and H.Y.).

7

8 **Abbreviations**

9 β 2M, β 2-microglobulin; CCCP, carbonyl cyanide m-chlorophenyl
10 hydrazine; COXII, cytochrome c oxidase subunit II; DCFDA,
11 2',7'-dichlorofluorescein diacetate; DCPIP,
12 2,6-dichlorophenolindophenol; ESR, electron spin resonance;
13 ETC, electron transport chain, Ir, ionizing radiation; LiNc-BuO,
14 lithium
15 5,9,14,18,23,27,32,36-octa-n-butoxy-2,3-naphthalocyanine;
16 mtDNA, mitochondrial DNA; MSR, MitoSOX Red; 3-NP,
17 3-nitropropionic acid; ROS, reactive oxygen species; TMRM,
18 tetramethylrhodamine methyl ester

19

20

21

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21

1 **Figure Legends**

2 Fig. 1. Flow cytometric analysis of cellular oxidative stress
3 levels and mitochondrial ROS levels in A549 cells after Ir. (A
4 and B) Cellular oxidative stress levels measured by DCFDA. (A)
5 Representative flow cytometric profiles obtained from the cells
6 after 0 and 12 h after Ir. (B) Time course of the relative
7 cellular oxidative stress levels. (C and D) Mitochondrial ROS
8 levels measured by MSR. (C) Representative flow cytometric
9 profiles obtained from the cells after 0 and 12 h after Ir. (D)
10 Timecourse of the relative mitochondrial ROS levels. Data are
11 expressed as means \pm SE of three experiments. * $p < 0.05$; ** p
12 < 0.01 vs. 0 h (Student's t -test).

13

14 Fig. 2. Flow cytometric analysis of mitochondrial membrane
15 potential in A549 cells after Ir. (A) Representative flow
16 cytometric profiles obtained from the cells after 0 and 12 h
17 after Ir. (B) Time course of the relative membrane potential.
18 Data are expressed as means \pm SE of four experiments. * $p < 0.05$;
19 ** $p < 0.01$ vs. 0 h (Student's t -test). (C) Effect of CCCP on
20 mitochondrial membrane potential. Data are expressed as means
21 \pm SE of three experiments. * $p < 0.05$ (Student's t -test).

22

23 Fig. 3. Oxygen consumption in A549 cells after Ir measured by
24 polarography. (A) Representative polarograph traces. The

1 oxygen concentration was monitored in the presence (0 h and 12
2 h) and absence (buffer only) of cells. Rotenone (2 μ M) was added
3 to stop mitochondrial respiration. (B) Oxygen consumption rate
4 in A549 cells after Ir. Data are expressed as means \pm SE of three
5 experiments. *p < 0.05 (Student's *t*-test).

6

7 Fig. 4. Oxygen consumption in A549 cells after Ir measured by
8 ESR oxymetry. (A) Representative ESR spectra obtained from
9 non-irradiated (left) or irradiated (right) A549 cells. (B)
10 Changes in pO₂ in A549 cells after Ir. After irradiation, A549
11 cells were incubated for 0 (●), 6 (○), 12 (■), or 24 (□) hours,
12 followed by the ESR measurements. Representative plots of three
13 experiments are shown. (C) Oxygen consumption rates in A549
14 cells after Ir. Data are expressed as means \pm SE of three
15 experiments. *p < 0.05 vs. 0 h (Student's *t*-test). (D) Effect
16 of rotenone on changes in pO₂ in A549 cells with and without
17 Ir exposure. Representative plots of three experiments are
18 shown. ●; Ir(-)/rotenone(-), ■; Ir(-)/rotenone(+), ○;
19 Ir(+)/rotenone(-), □; Ir(+)/rotenone(+). (E) Effect of
20 rotenone on cellular oxygen consumption. Data are expressed as
21 means \pm SE of three experiments. *p < 0.05; **p < 0.01 (Student's
22 *t*-test).

23

24 Fig. 5. Cellular ATP content in A549 cells after Ir. (A) Time

1 course of the relative cellular ATP content. After 10 Gy
2 irradiation, A549 cells were collected and cellular ATP content
3 was measured as described in the text. The ATP amounts at each
4 time point were normalized to the ATP amount at 0 h after Ir.
5 (B) Effect of oligomycin on cellular ATP content after Ir.
6 Immediately after irradiation, A549 cells were incubated with
7 oligomycin for 12 h. The ATP amounts in each condition were
8 normalized to the ATP amount obtained from Ir(-)/oligomycin(-)
9 cells. Data are expressed as means \pm SE of four experiments.
10 *p < 0.05 vs. 0 h (Student's *t*-test).

11

12 Fig. 6. Effect of the complex II inhibitor 3-nitropropionic acid
13 (3-NP) on mitochondrial ROS level after Ir. After A549 cells
14 were X-irradiated, the cells were incubated with 3-NP at the
15 indicated concentrations for 12 h. Intensities of MSR
16 fluorescence in each condition were normalized to those
17 obtained from Ir(-)/3-NP(-) cells. Data are expressed as means
18 \pm SE of three experiments.

19

20 Fig. 7. Mitochondrial content in A549 cells after Ir. (A)
21 Mitochondrial mass measured by MitoTracker Green staining.
22 After 10 Gy irradiation, A549 cells were stained with
23 MitoTracker Green and analyzed using a flow cytometer.
24 Representative flow cytometric profiles of three experiments

1 are shown. (B) Mitochondrial DNA (mtDNA) content measured by
2 quantitative PCR. The relative amounts of D-loop and cytochrome
3 c oxidase subunit II (COXII) in total DNA were determined as
4 described in the text. MtdNA content at each time point was
5 normalized to that at 0 h after Ir. Data are expressed as means
6 \pm SE of three experiments. * $p < 0.05$ vs. 0 h (Student's *t*-test).

7

8 Fig. 8. Simultaneous flow cytometric analysis of cell
9 cycle/mitochondrial mass in A549 cells after Ir. After 10 Gy
10 irradiation, A549 cells were double-stained with Nuclear-ID Red
11 and MitoTracker Green, followed by the flow cytometric analysis.
12 (A) Representative flow cytometric profiles in A549 cells after
13 Ir analyzed by Nuclear-ID Red fluorescence. (B) MitoTracker
14 Green fluorescence of the cell fraction corresponding to the
15 G1, S, and G2/M phases. Representative flow cytometric profiles
16 of MitoTracker Green fluorescence from each cell cycle fraction
17 at the indicated times after Ir are overlaid and shown. white;
18 G1, black; S, gray; G2/M. (C) Relative mitochondrial content
19 during the cell cycle after Ir. Data are expressed as means \pm
20 SE of three experiments. ** $p < 0.01$ vs. 0 h (Student's *t*-test).

21

22 Fig. 9. Simultaneous flow cytometric analysis of cell
23 cycle/cellular oxidative stress levels in A549 cells after Ir.
24 After 10 Gy irradiation, A549 cells were double-stained with

1 Nuclear-ID Red and DCFDA, followed by the flow cytometric
2 analysis. (A) Representative flow cytometric profiles in A549
3 cells after Ir analyzed by Nuclear-ID Red fluorescence. (B)
4 DCFDA fluorescence of the cell fraction corresponding to the
5 G1, S, and G2/M phases. Representative flow cytometric profiles
6 of DCFDA fluorescence from each cell cycle fraction at the
7 indicated times after Ir are overlaid and shown. white; G1,
8 black; S, gray; G2/M. (C) Relative cellular oxidative stress
9 levels during the cell cycle after Ir. Data are expressed as
10 means \pm SE of three experiments. **p < 0.01 vs. 0 h (Student's
11 t-test).

Table 1

Mitochondrial ETC enzyme activities after Ir

Condition	Complex I+III	Complex II+III	Complex IV	Complex I	Complex II
Ir (-)	27.8 ± 6.4	58.5 ± 10.5	228.8 ± 65.5	23.8 ± 4.3	38.7 ± 8.1
Ir (+)	26.3 ± 3.3	74.5 ± 15.8	216.1 ± 63.2	23.8 ± 8.4	61.8 ± 16.8

Complex I+III, complex II+III, and complex IV activities are expressed in nmol cytochrome c (reduced or oxidized)/min • mg protein. Complex I activities are expressed in nmol NADH/min • mg protein. Complex II activities are expressed in nmol DCPIP/min • mg protein. Data are expressed as means ± SE of at least five experiments.

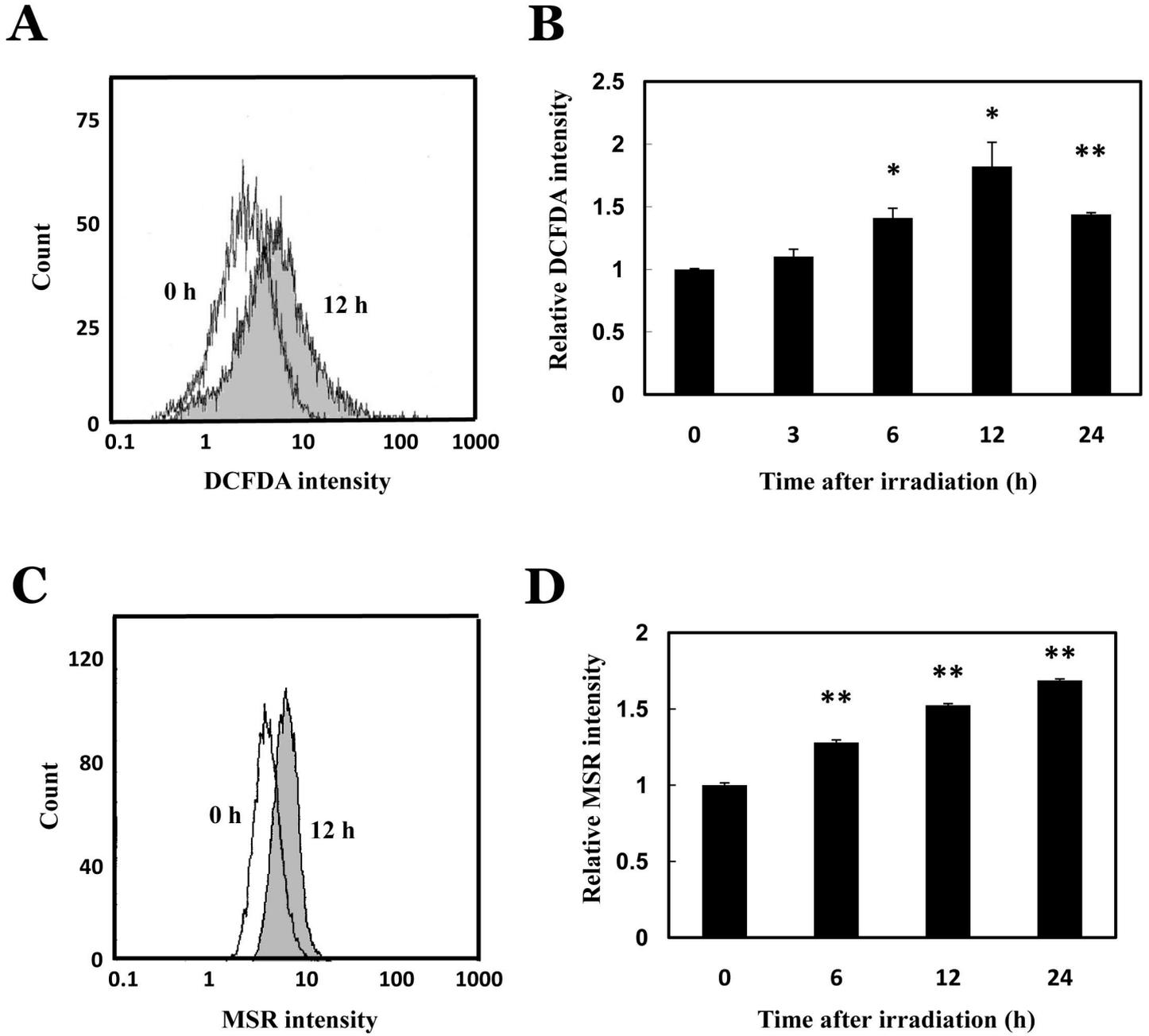


Fig. 1 Yamamori *et al.*

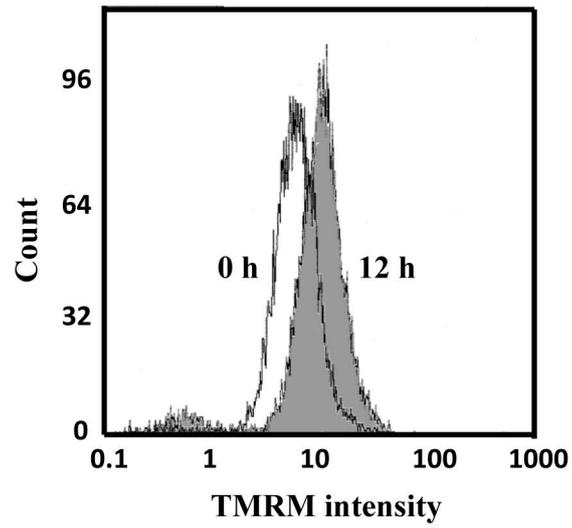
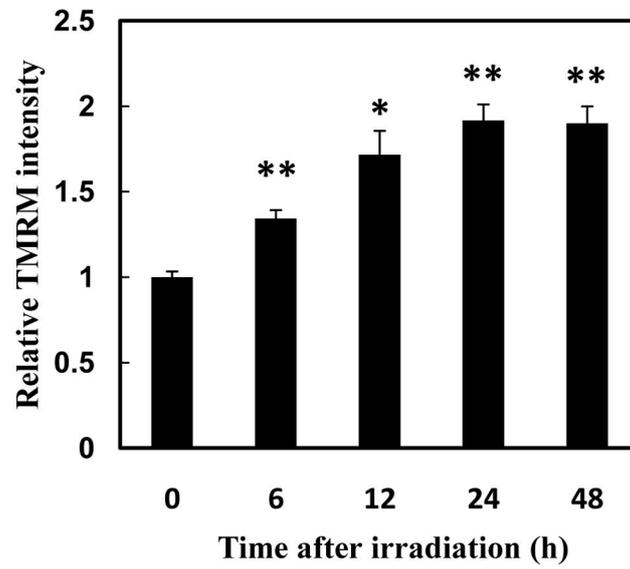
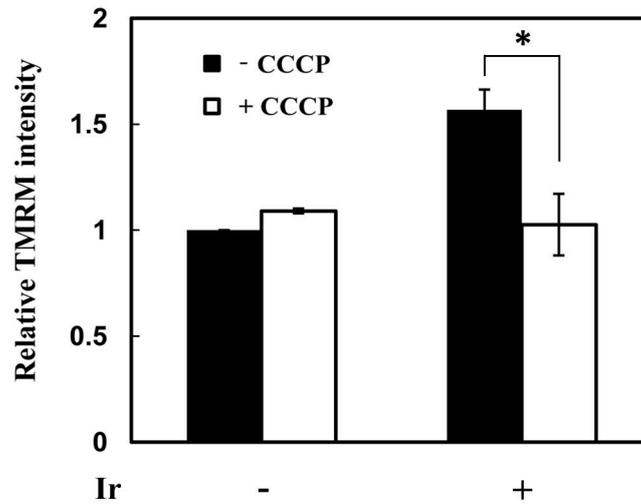
A**B****C**

Fig. 2 Yamamori *et al.*

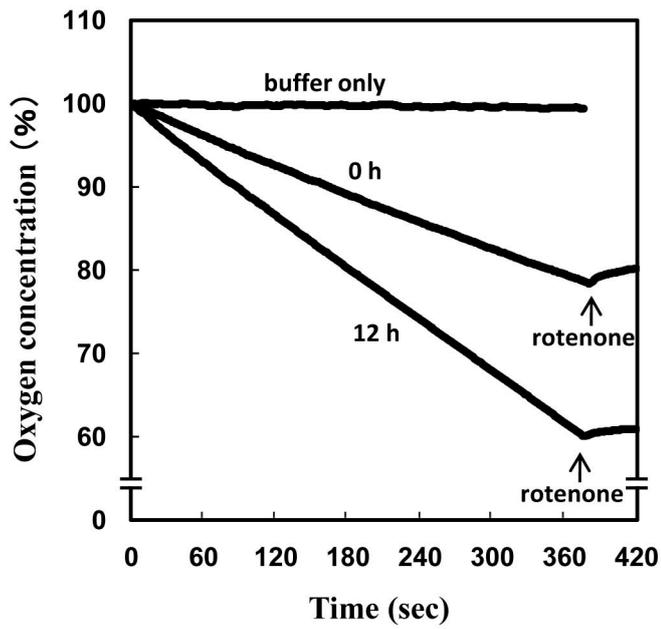
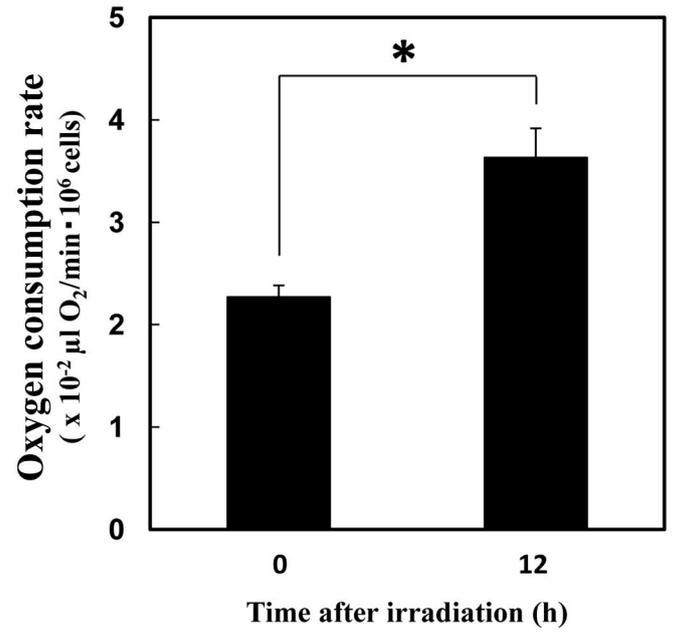
A**B**

Fig. 3 Yamamori *et al.*

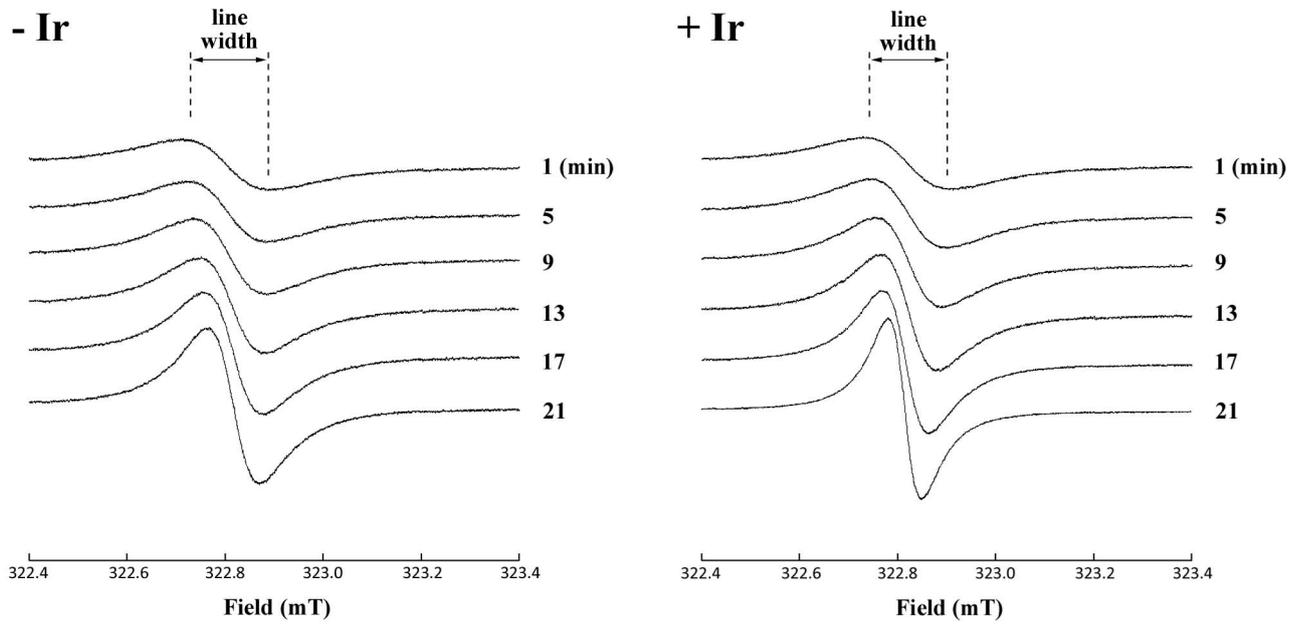
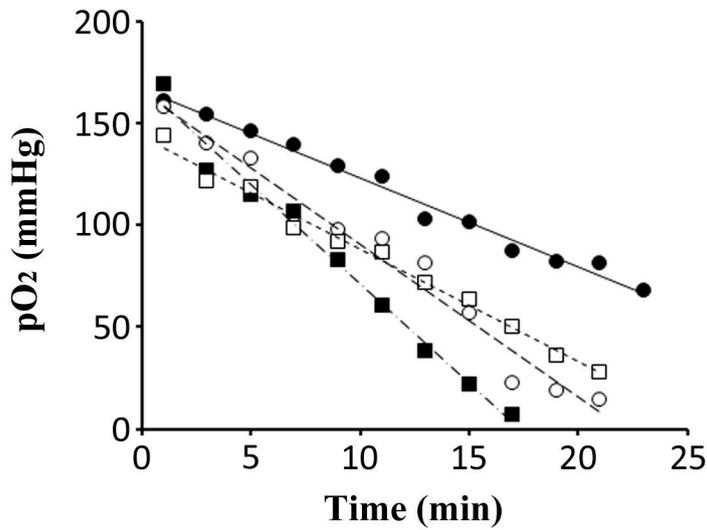
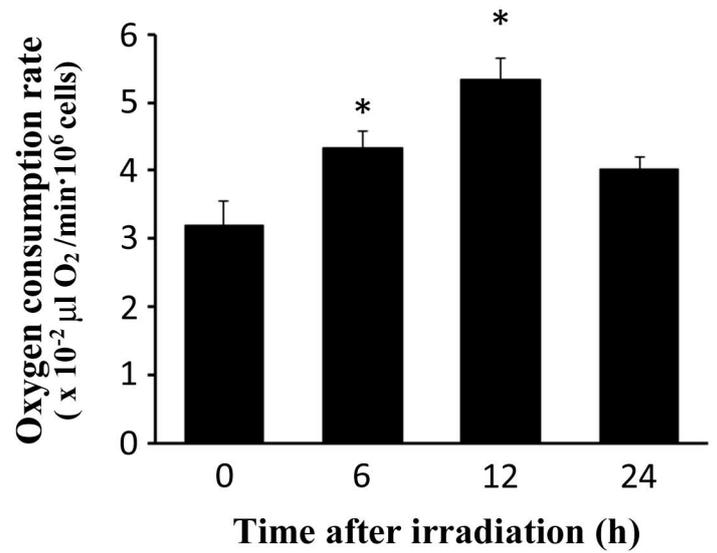
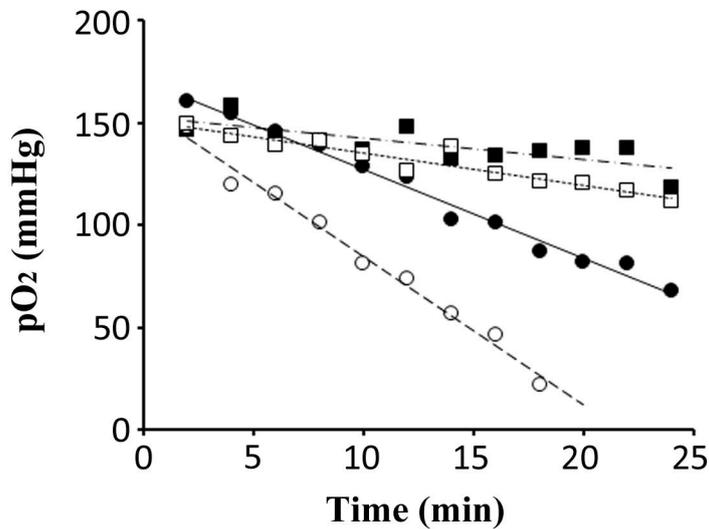
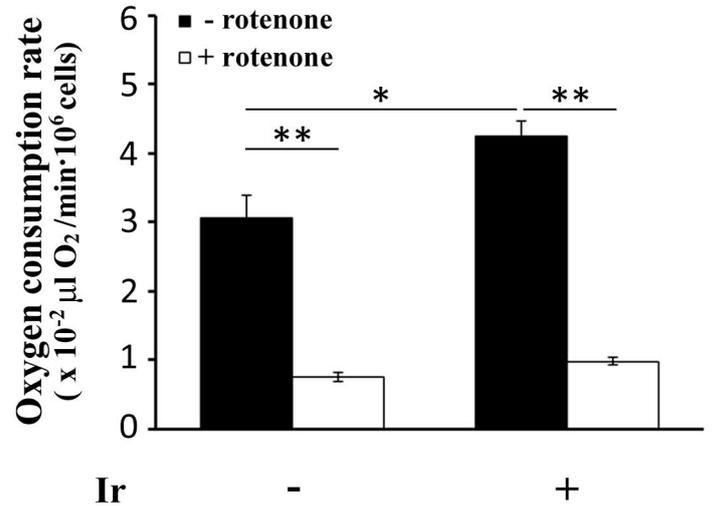
A**B****C****D****E**

Fig. 4 Yamamori *et al.*

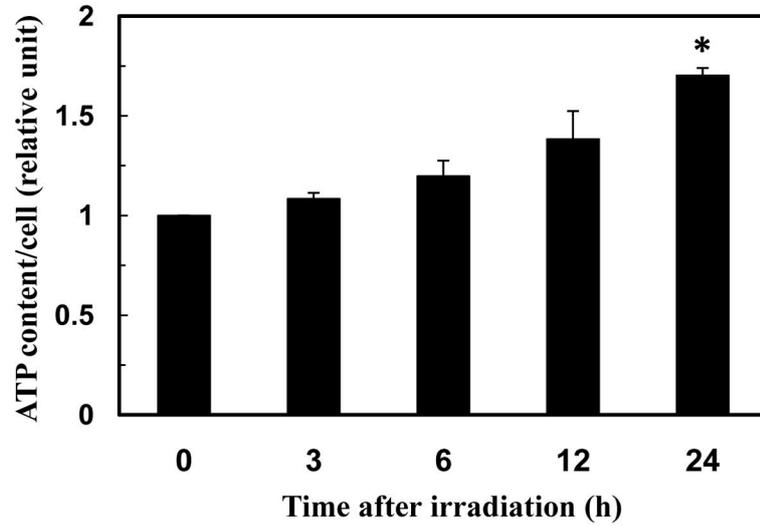
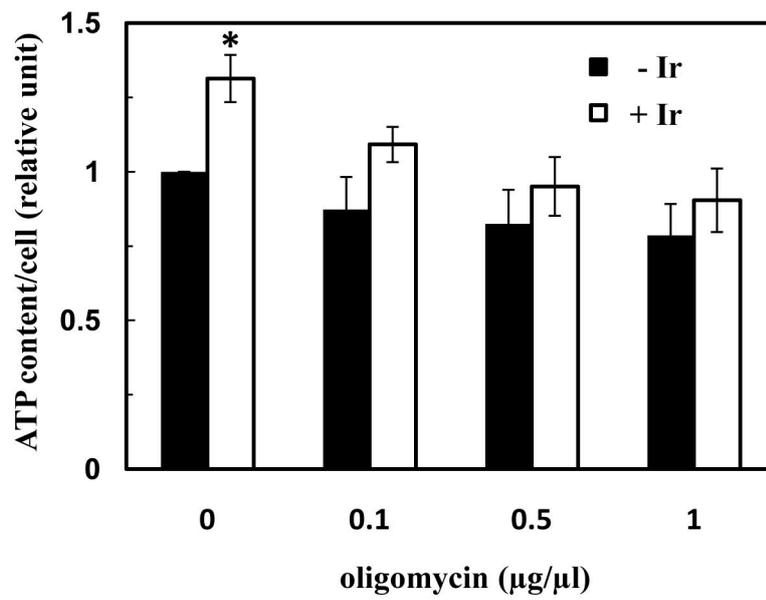
A**B**

Fig. 5 Yamamori *et al.*

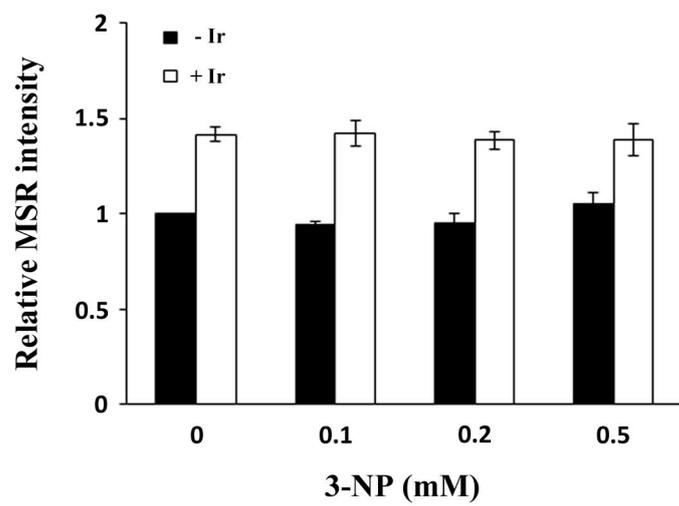


Fig. 6 Yamamori *et al.*

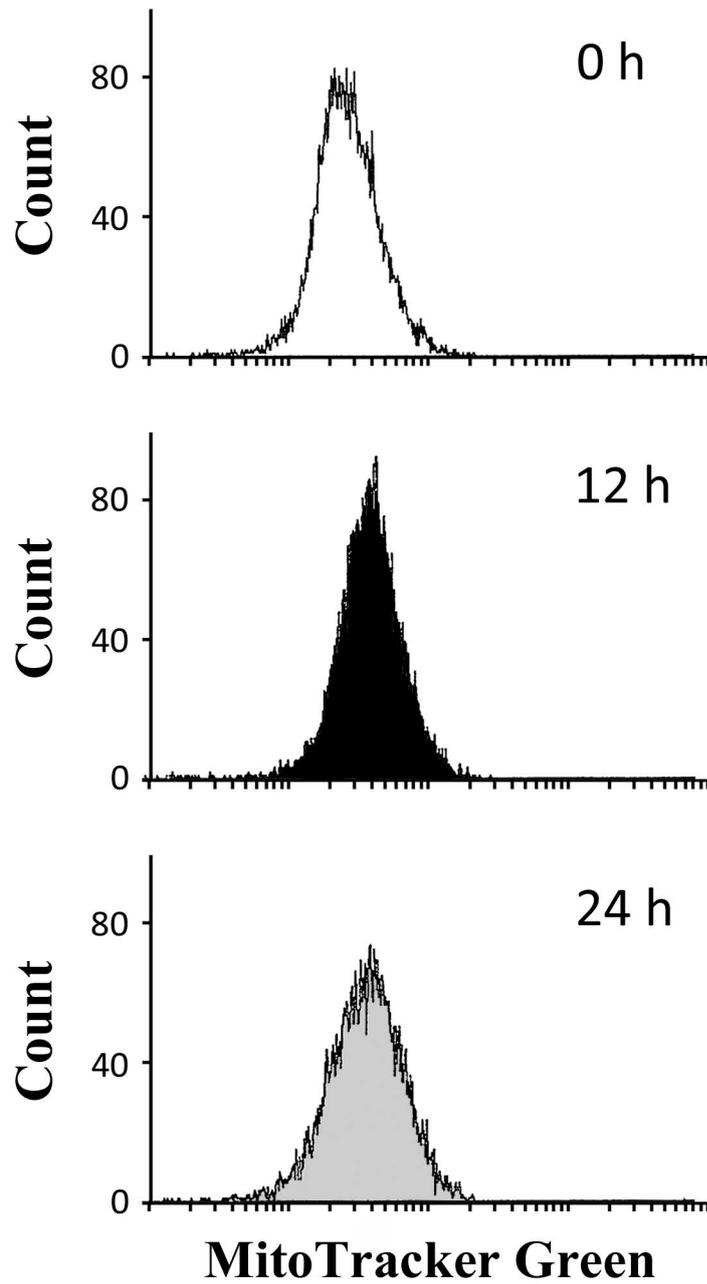
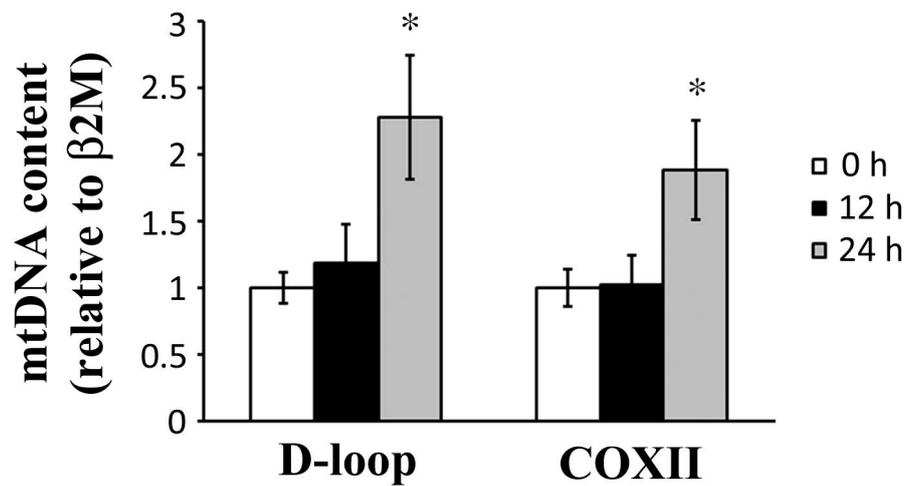
A**B**

Fig. 7 Yamamori *et al.*

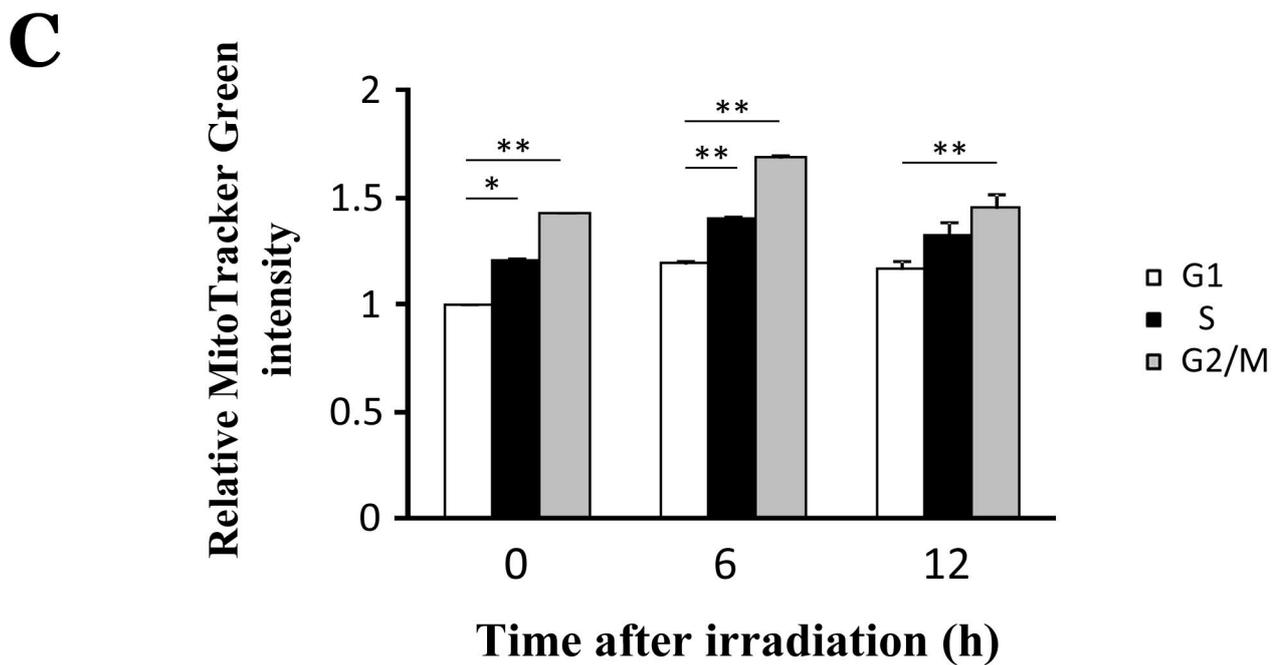
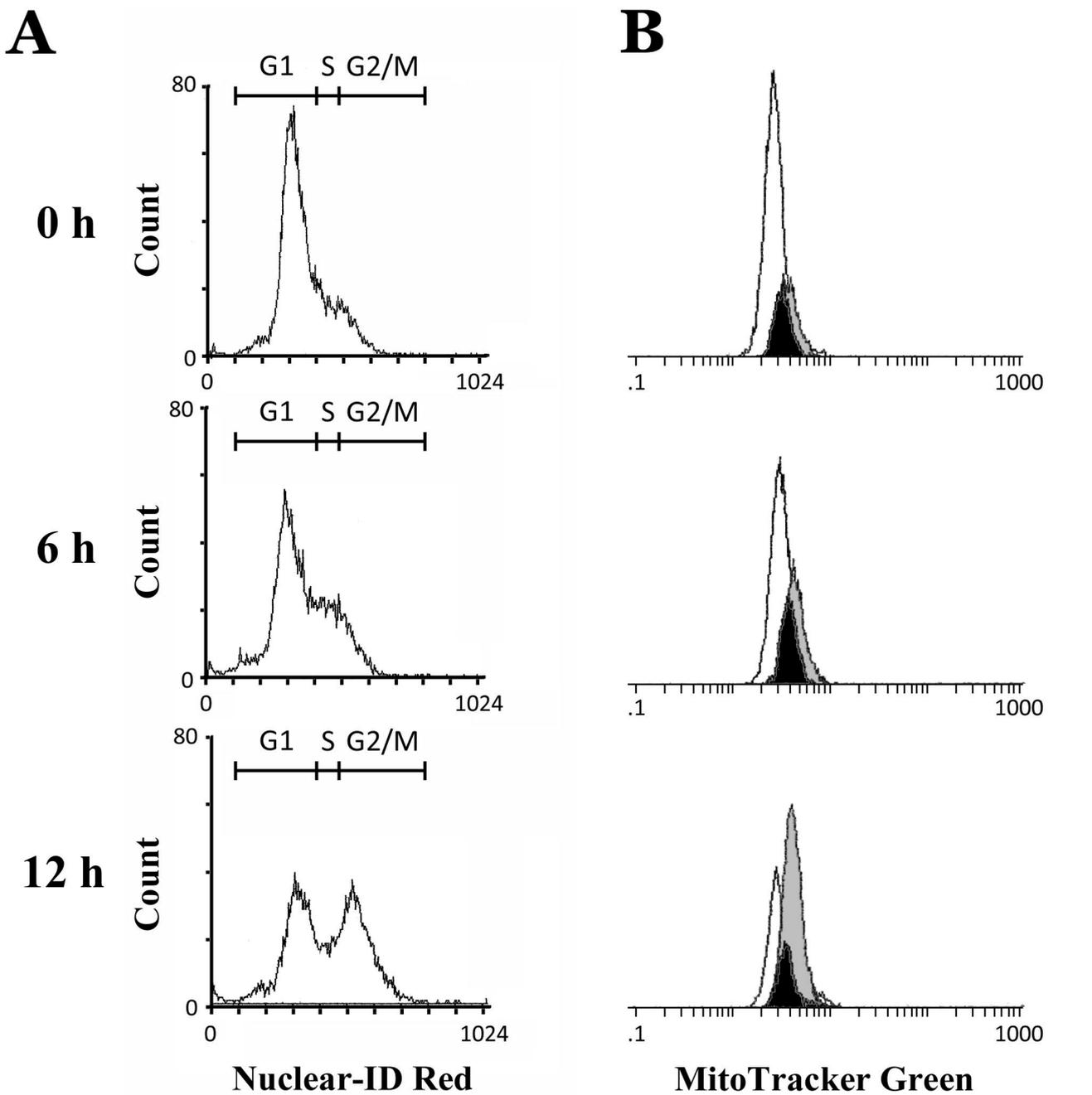


Fig. 8 Yamamori *et al.*

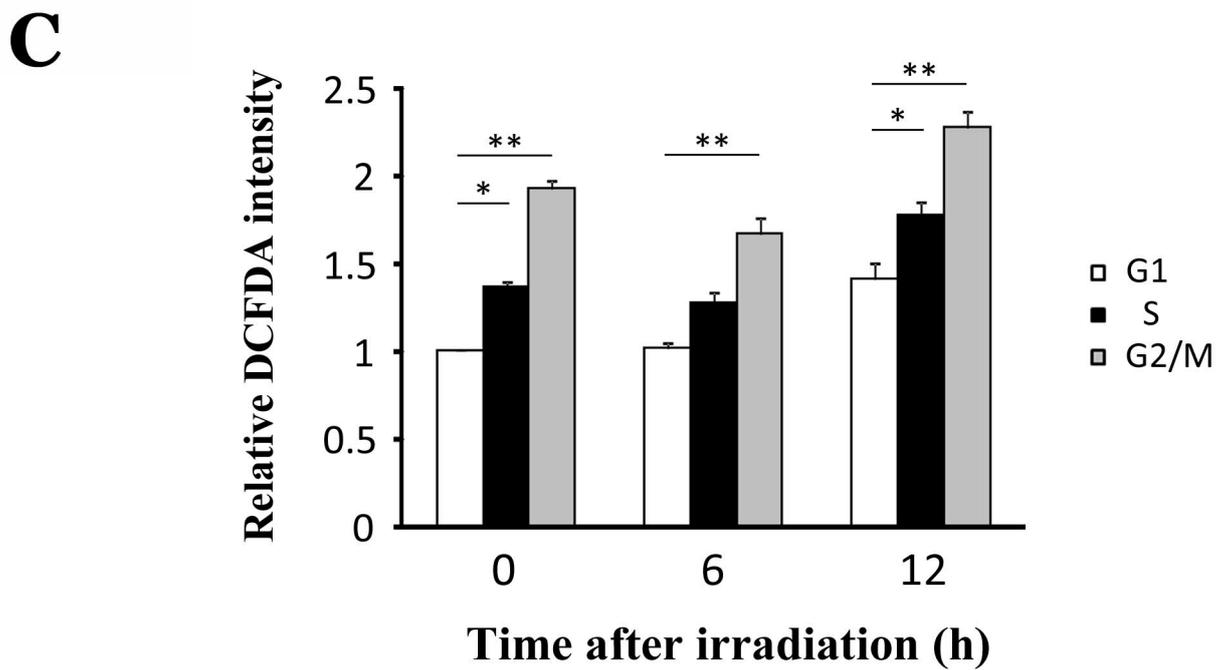
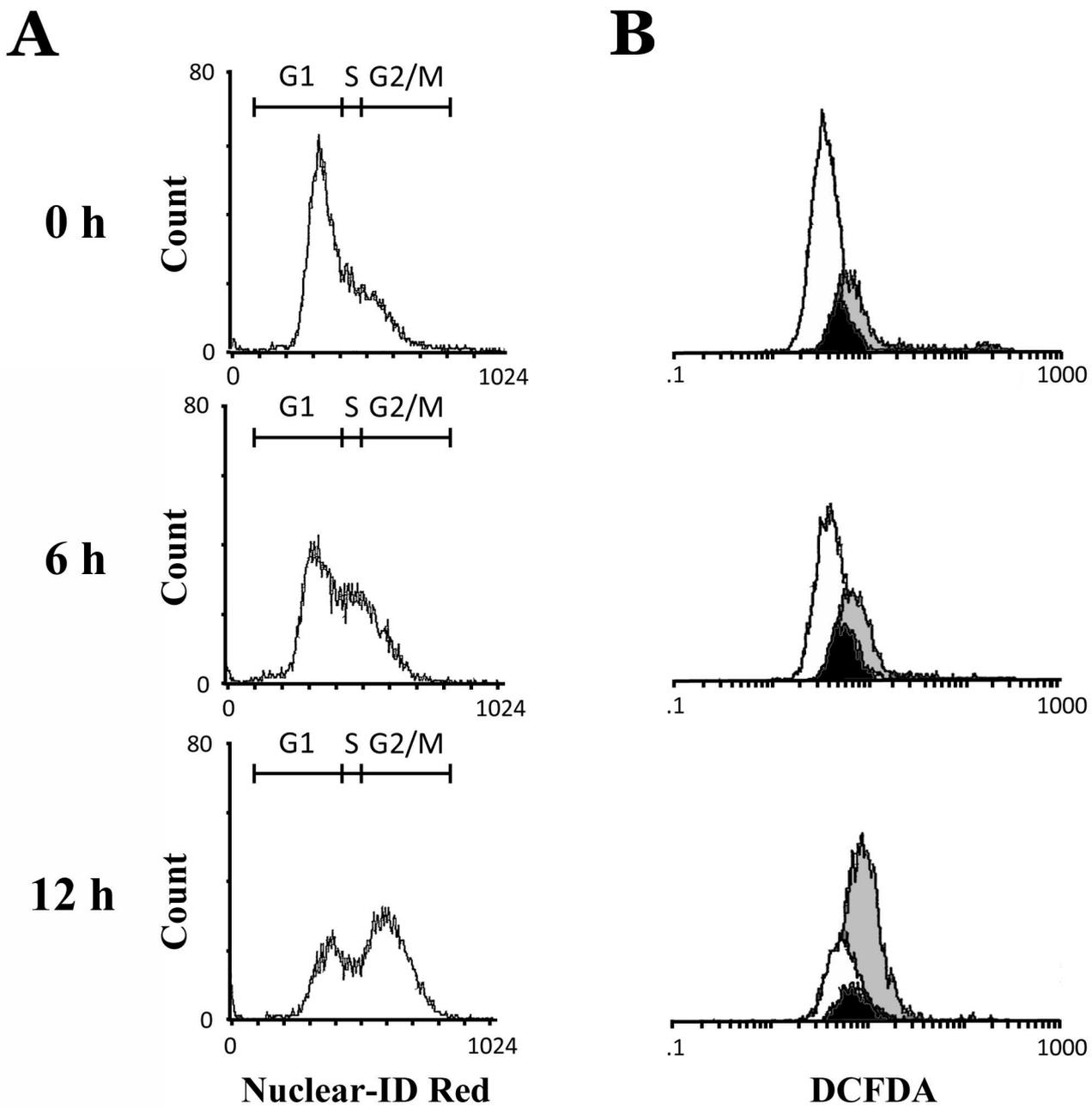


Fig. 9 Yamamori *et al.*