Protection against radiation-induced damage of 6-propyl-2-thiouracil (PTU) in thyroid cells

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Abstract

Introduction: Many epidemiologic studies have shown that the exposure to high external radiation doses increases thyroid neoplastic frequency, especially when given during childhood or adolescence. The use of radioprotective drugs may decrease the damage caused by radiation therapy and therefore could be useful to prevent the development of thyroid tumors. The aim of this study was to investigate the possible application of 6-propyl-2-thiouracil **(**PTU) as a radioprotector in the thyroid gland.

*Materials and Methods***:** Rat thyroid epithelial cells (FRTL-5) were exposed to different doses of γ-irradiation with or without the addition of PTU, methimazole (MMI), reduced glutathione (GSH) and perchlorate ($KClO_4$). Radiation response was analysed by clonogenic survival assay. Cyclic AMP levels were measured by radioimmunoassay **(**RIA). Apoptosis was quantified by nuclear cell morphology and caspase-3 activity assays. Intracellular reactive oxygen species (ROS) levels were measured using the fluorescent dye 2', 7'-dichlorofluorescein-diacetate. Catalase, superoxide dismutase and glutathione peroxidase activities were determined.

Results: Pretreatment with PTU, MMI and GSH prior to irradiation increased significantly the surviving cell fraction (SF) at 2 Gy ($P < 0.05$) while no effect was observed with $KClO_4$. An increase in extracellular levels of cAMP was found only in PTU treated cells, in a dose and time-dependent manner. Cells incubated with agents that stimulate cAMP (Forskolin and dibutyril cAMP), mimicked the effect of PTU on SF. Moreover, pretreatment with the inhibitor of protein kinase A, H-89, abolished the radioprotective effect of PTU. PTU treatment diminished radiation-induced apoptosis and protected cells against radiation-induced ROS elevation and suppression of antioxidant enzyme's activity.

Conclusion: PTU was found to radioprotect normal thyroid cells through cAMP elevation. There was also a reduction in apoptosis levels and in radiation-induced oxidative stress damage.

Introduction

Ionizing radiation is commonly used in the treatment of cancer, but the amount of radiation that can be delivered to the tumor to treat it efficiently, is often limited by the toxicity to organs and surrounding tissues. Radioprotectors are chemical compounds that protect normal cells from the damage caused by radiation therapy (1, 2). Patt et al. (3) found that pretreatment of rats with cysteine reduced radiation-induced lethality. Therefore, during the past decades there has been a constant effort worldwide to find an agent to protect normal tissue during the application of radiation therapy (4, 5). Most of them are sulfhydryl compounds and although they decrease the effects of radiation *in vitro*, their clinical use is reduced due to their toxic effects. Amifostine, a thiol-containing prodrug, is the only drug that has been approved by the Food and Drug Administration (FDA, USA) for xerostomia in patients receiving radiotherapy for head and neck cancers.

Epidemiologic studies have shown that the exposure to high external radiation doses increases thyroid neoplastic frequency, especially when given during childhood or adolescence (6-10). Different studies showed that patients bearing benign or malignant conditions who have been treated with radiation developed thyroid cancer. Moreover the risk of hypothyroidism increases proportionally with the dose of radiation received (11, 12), while the risk of developing thyroid malignancies follows a linear-dose response and decreases with higher doses of radiation (> 10 Gy) (13). The application of a radioprotective drug to the thyroid gland could be useful to avoid the development of malignancies in the neck area after radiation treatment, specifically for diseases that are

often treated by radiotherapy. Some of them include the Hodgkin lymphoma and tumors of the lymphatic system (14).

Antithyroid compounds, such as methylmercaptoimidazole (MMI) and propylthiouracil (PTU), are thioureylene drugs that have been used to treat hyperthyroidism since 1940. They inhibit the thyroperoxidase enzyme, reducing then the synthesis of thyroid hormones. PTU also inhibits the peripheral conversion of thyroxine to triiodothyronine. Like many radioprotective substances, these compounds share a simple chemical structure containing a sulphur atom (15). Greig et al. (16-7) showed that MMI protected rat thyroid gland from radiation injury and recently it was shown that after external irradiation of thyroid cells, DNA damage was reduced by MMI to 70% (18).

In the present work we investigated the possible role of PTU as a radioprotector in the thyroid gland and its mechanism of action. This mechanism of radioprotection could be exerted directly trough free radical scavenging or indirectely by modifying intracellular levels of cyclic AMP.

Materials and Methods

Cell Culture

Fisher rat thyroid (FRTL-5) cell line was cultured in Dulbecco's modified Eagle medium (DMEM/F12, 50:50 v/v) (GIBCO, Invitrogen Corporation, USA) supplemented with 5% fetal bovine serum (FBS) (Natocor, Córdoba, Argentina), bovine thyrotropin (1 mU/mL), hydrocortisone (3.62 ng/mL), transferrin (5μg/mL), insulin (10 mg/mL), somatostatine (10

ng/ml) and glycil-L-histidyl-L-lysine acetate (10 ng/mL) (Sigma, St Louis, MO, USA). Cell cultures were kept at 37 ºC in 5 % CO_2-95% air atmosphere in a humidified incubator.

Irradiation Characteristics

Cells were irradiated with a cobalt gamma ray source (Institute of Oncology "Ángel Roffo", Buenos Aires, Argentina, 1 Gy/min ± 5%) for different times in order to obtain radiation absorbed doses ranged between 1 and 8 Gy. Corresponding controls were sham irradiated.

Clonogenic Assay

Exponentially growing cells were distributed into the following groups: a) irradiated without treatment, and b) incubated with the drug before irradiation. Cells were seeded in T25 flasks 24 hours before the irradiation and incubated with different treatments: 1 mM PTU, 0.01 mM Forskolin, 1 mM dibutyril cAMP (dbcAMP), 1 mM PTU + 0.01 mM H-89, 1 mM potassium perchlorate ($KClO_4$), 1 mM MMI or 1 mM reduced glutathione (GSH). After the irradiation, cells were harvested, counted and seeded in 60-mm culture plates at different densities according to the radiation dose received and were replaced into the incubator to allow colony formation. 9-14 days later, colonies were fixed with 5% glutaraldehyde and stained with 0.5% crystal violet. Colonies with more than fifty cells were counted manually. The surviving fraction of each radiation dose was normalized to that of the control to obtain a radiation survival curve. Curves were fitted according to the linear-quadratic model (surviving fraction = $\exp^{-\alpha(D)-\beta(D)2}$) using Origin 7.5 (OriginLab, USA). Surviving fraction at 2 Gy (SF2) was calculated from each curve.

Cyclic AMP Measurement

Cells were seeded in 24-well culture plates and were incubated with different concentrations of PTU (0-1 mM), with or without the addition of the phosphodiesterase inhibitor 100 μM 1-methyl-3-isobutylxanthine (IBMX) for different lengths of time (6, 24, 48 and 72 hours). Cells were incubated also with MMI, $KClO_4$ or GSH at the same dose (1 mM) for 24 hours. After the treatment, the medium was collected for extracellular measurement. Cyclic AMP (cAMP) was determined using the method described previously (19). The antibodby used was provided by the NIH (National Hormone and peptide program, Dr A.F. Parlow, NIH, USA). Results are expressed in fmol cAMP/μg protein. The interassay and intraassay variations of coefficients were lower than 10%.

Intracellular ROS levels

Intracellular reactive oxygen species (ROS) levels were determined 1 and 24 hours after irradiation using the fluorescent dye 2', 7'-dichlorofluorescein-diacetate (DCFH-DA). DCFH-DA diffuses passively inside the cell where it is deacetylated by intracellular esterases to a nonfluorescent polar product (DCFH). In the presence of hydrogen peroxide, the derivative is oxidized to the highly fluorescent 2', 7'-dichlorofluorescein (DCF). Cells were incubated with 10 μM of DCFH-DA at 37 ºC for 20 minutes and immediately after ROS levels were determined by fluorescence at λexcitation: 485/20 nm and λemission: 530/25 nm using a plate reader (Beckman Coulter DTX 880 Multimode detector; Beckman Coulter, Inc., CA, USA). An aliquot of the cells was separated for protein determination. Results are expressed as relative absorbance units/mg protein.

Enzymatic Assays

For determination of enzymatic activities, cells were washed and scraped with PBS and spun at 1200 x g for 10 min at 4 ºC 1 and 24 hours after irradiation. The pellets were resuspended in PBS and sonicated for two 15-s bursts. Sonicates were centrifuged for 10 min at 15000 x g at 4 ºC and the resulting supernatants were collected for enzymatic and protein determination.

Catalase

The activity was measured by monitoring spectrophotometrically the disappearance of H_2O_2 at 240 nm catalyzed by the enzyme by the method described by Aebi (20). Activity is expressed as nmol of H_2O_2 consumed per minute per mg protein.

Glutathione peroxidase

Enzymatic activity was determined indirectly trough the decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to $NADP^+$ with the Glutathione Peroxidase Cellular Activity Assay Kit (SIGMA CGP-1). A unit of glutathione peroxidase is defined as the amount of enzyme that will cause the formation of 1.0 μmol of $NADP^+$ from NADPH per minute at pH 8.0 at 25 ºC in the presence of reduced glutathione, glutathione reductase and H_2O_2.

Superoxide Dismutase

Superoxide dismutase (SOD) enzymatic activity was measured using a SOD Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). Assay was performed according to the manufacturer's instructions and absorbance was measured at 450 nm. Results are expressed as percentage of unirradiated controls.

Cell death assessment by nuclear morphology

Apoptosis was assessed using fluorescence staining 6, 24, 48 and 72 hours after irradiation. Cells were incubated with 2 µl of a MIX buffer containing Hoechst 33258 (0.59 mg/mL), 4, 5-diaminofluorescein (DAF) (0.12 mg/mL) and Propidium Iodide (PI) (0.59 mg/mL) at 37ºC for five minutes. Propidium Iodide and DAF were used to stain dead cells and cytoplasm of viable cells, respectively. Hoechst 33258 was used to evaluate nucleus' morphology and differentiate apoptotic nuclei from normal ones. An epifluorescence microscope (Olympus BX51) was used and serially pictures were taken using a CCD capture camera (Olympus DP70). The percentage of apoptotic cells was determined by identifying those cells with chromatin condensation and fragmentation characteristics of apoptosis over at least 200 observed cells.

Caspase-3 activity

Cells were collected in PBS and centrifuged at 600 x g for 5 min at 4 ºC. The pellet was resuspended in 1 X lysis buffer (250 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid [HEPES], pH 7.4, 25 mmol/L 3[3-cholaminopropyl diethylammonio]-1-propane sulfate [CHAPS], 25 mmol/L dithiothreitol [DTT]), incubated in ice for 15 minutes and centrifuged at 15000 x g for 20 min at 4 ºC. Caspase-3 activity was measured in the supernatant 6, 24, 48 and 72 hours after irradiation with the Caspase 3 Colorimetric Assay Kit (Sigma, CASP-3-C; Sigma-Aldrich, St. Louis, Mo, USA). Briefly, a volume containing 200 µg of protein was placed in a 96-well plate and incubated with the caspase-3 substrate (2 mM Ac-DEVD-pNA), at 37 ºC for 90 min. The concentration of the p-nitroaniline released from the substrate is calculated from the absorbance values at 405 nm after the incubation. The activity (µmol pNA released per min per ml) was calculated with a

p-nitroaniline calibration curve. A positive control of caspase-3 and an inhibitor of caspase-3 (200 μM Ac-DEVD-CHO) were added to the plate.

Statistical Analysis

Differences between surviving fractions were calculated with two tailed Student's *t* test. Differences among experimental groups were determined using one way ANOVA, followed by Tukey-Kramer multiple comparison test. Data are expressed as mean ± SE. Experiments were reproduced at least three times. Differences were considered significant at $p < 0.05$.

Results

Radioprotective effect of PTU

Exponentially growing cell cultures were pretreated with 1 mM PTU or the same volume of medium for 24 hours before the irradiation. The selected dose of the drug did not affect the proliferating rate (results not shown). As shown in Figure 1A, PTU caused an increase in post-irradiation survival of FRTL-5 cells (SF2: Control: 56.9 ± 3.1; PTU: 75.0 ± 4.4; *$p < 0.05$). When PTU is added after irradiation no protection was observed (data not shown), suggesting that the presence of the drug during the irradiation is critical for the radioprotective effect. To assess the specificity of the studied effect, we incubated cells with other molecular related species (MMI and GSH) and with perchlorate a competitive inhibitor of the sodium iodide symporter (NIS). Figure 1B shows that cell pretreatment with MMI and GSH increased SF after irradiation (SF2: Control 50.5 ± 4.1; MMI: 74.6 ± 5.2; GSH 81.8 ± 5.5, *$p < 0.05$). On the other hand, we did not observe a radioprotective

effect in the cells incubated with perchlorate (SF2, Control: 53.4 ± 3.5; $KClO_4$: 58.9 ± 6.1) (Fig.1C).

Effects of PTU on cyclic AMP levels

In order to explore the possible mechanism of action of PTU, cAMP levels were measured at different lengths of time. Table 1 A shows that after 5 and 24 hours, extracellular cAMP levels were significantly increased in cells treated with PTU at both doses (** $p < 0.01$). Groups treated with PTU plus IBMX showed a significant increase only at 5 hours (** $p < 0.01$).

To further evaluate if the other compounds could elevate cAMP levels as well, we incubated FRTL-5 cells for 24 hours at the same dose used to test the survival fraction after irradiation. Extracellular cAMP levels were only significantly increased in cells incubated with PTU (** $p < 0.01$) and no significant differences between groups were found with the addition of IBMX (Table 1 B).

As PTU increased cAMP levels, we studied the effects of diverse cAMP increasing agents. We observed that SF2 was increased from 58.0 ± 2.5 to 67.8 ± 0.4 in cells treated with 1 mM dbcAMP (** $p < 0.01$) (Fig. 2A). SF at higher irradiation doses was not significantly modified. On the other hand, Forskolin increased SF2 from 55.8 ± 0.8 to 71.1 ± 3.9 (** $p < 0.01$) in FRTL-5 cells; for higher doses the increase in the survival fraction was also significant (Fig. 2B). To further test the possibility of PTU acting as a radioprotective drug through cAMP elevation, we incubated cells with PTU and the protein kinase A inhibitor H-89. We found that H-89 abolished the radioprotective effect of PTU in FRTL-5 cells (SF2, Control: 53.3 ± 3.9; PTU: 74.3 ± 6.5; PTU + H-89: 57.9 ± 5.3, * $p < 0.05$ *vs* PTU) (Fig. 2C).

Effect of PTU on oxidative stress and radiation-induced apoptosis

Incubation with PTU alone did not cause a variation in ROS levels compared to untreated controls. Irradiation alone induced a dose-dependent increase of approximately 1.2 and 1.4-fold in FRTL-5 cells 1 hour after irradiation, at 3 and 5 Gy respectively. No significant effect of PTU was observed (Fig. 3). There was not variation in ROS levels 24 hours after irradiation.

The activity of different anti-oxidant enzymes was evaluated in irradiated and unirradiated cells 1 and 24 hours after irradiation at 3 and 5 Gy. Treatment with PTU lead to an increase of SOD activity at both doses after 1 hour in irradiated cells (** $p < 0.01$ for 3 Gy and * $p < 0.05$ for 5 Gy) (Fig. 4A). At 24 hours there was no irradiation or PTU effect on SOD activity. 1 hour after irradiation, catalase activity was increased at 3 Gy (* $p < 0.05$) (Fig. 4B). At 24 hours, PTU pretreatment increased the enzyme activity at all doses studied (* $p < 0.05$). GPX activity could not be detected in FRTL-5 cells at both assayed times (results not shown).

Cell irradiation induced apoptosis and necrosis, as evidenced by morphological features of programmed cell death like chromatin condensation, cytoplasm vacuolization and apoptotic bodies. The percentage of dead cells was higher than that of apoptotic cells. The ratio dead/apoptotic cells decreased with higher doses of radiation except at early times of irradiation. PTU diminished the percentage of apoptotic and dead cells especially at late times (Fig. 5A and 5B, respectively).

Caspase-3 activity, an indicator of apoptosis, was significantly increased in irradiated cells at later times. PTU diminished the enzyme activity only at 3 Gy 24 and 48 hours after irradiation (Fig. 6).

Discussion

Radiotherapy is one of the most common therapies used for the treatment of cancer but it causes collateral damage to the surrounding tissues. Several drugs have been tested as radioprotectors in order to avoid healthy cells from radiation collateral effects, but their high toxicity excluded them from clinical use (1-4).

Thyroid function could be affected in patients treated with radiotherapy for head and neck cancer resulting in thyroid cell injury, immune-mediated damage and vascular injury (9-14, 21-2). Moreover the use of pediatric computerized tomographies (CT) has risen 8-fold since 1980 (23) and there is an increasing concern regarding radiation exposure to the thyroid gland due to this practice.

Antithyroid drugs, such as methylthiouracil, have been tested as radioprotectors for the thyroid gland. Greig et al. showed that pretreatment of rats with methylthiouracil before irradiation decreased radiation-induced damage in the gland (7, 8). PTU treatment for 15 days reduced damage in several organs *in vivo*. The authors suggested that hypometabolism, due to the hypothyroidism induced by PTU, could be the mechanism of such radioprotection (24). Some authors reported a radioprotective effect of antithyroid compounds in patients undergoing radioiodine treatment, although the effect could be related to a reduction of intrathyroid iodine half life due to a direct inhibition of thyroperoxidase (25-27). Also, a possible interaction of MMI with the sodium iodide symporter may not be ruled out. Kahmann et al. observed that in FRTL-5 cells following

external irradiation, DNA damage measured by the comet assay was reduced by methimazole to 70% (9).

The aim of this work was to further explore the possible radioprotective role of the anthithyroid drug PTU studying its mechanism of action. We chose to use the FRTL-5 normal rat thyroid cell line because it reproduces *in vitro* many aspects of the behavior of normal thyroid *in vivo*. We found that pretreatment of FRTL-5 cells with 1 mM PTU before γ-irradiation resulted in a significant increase in survival fraction at 2 Gy (39%) compared to control irradiated cells. Similar results were obtained with MMI and GSH but not with $KClO_4$. PTU, MMI and GSH contain a sulfhydryl group such as other radioprotective compounds (5). It has been shown that perchlorate, iodide, and thiocyanate protected FRTL-5 cells against DNA double-strand breaks induced by ^{131}I (28). The discrepancy with our results may be related to the fact that perchlorate is a well known inhibitor of iodide transport; on the other hand we studied the radioprotective effect against external irradiation.

Adenosine 3', 5'-cyclic monophosphate (cAMP) is a cyclic nucleotide present in all mammalian cells. Many studies have shown that cAMP regulates many cellular functions and growth under normal and abnormal conditions (29, 30). It has been described that factors like the proliferation rate, the degree of differentiation and the step in the cell cycle during the irradiation exposure may modify the survival of irradiated cells (31). Therefore the question is whether cAMP may be involved in this effect. Several reports have shown that an increase in cAMP is related to a radioprotective effect (32-37). However this role was discarded in the eicosanoid-induced radioprotection of bovine aortic endothelial cell

monolayers *in vitro* (38). To explore if a possible mechanism of action of PTU could be exerted by increasing cAMP we measured its levels. We found that extracellular cAMP was elevated in FRTL-5 cells incubated with different doses of PTU. MMI and GSH did not change cAMP levels as it was previously reported (39, 40). It has been shown that iodine, through the production of an intracellular organified iodine intermediate (41), could exert an inhibitory effect in cAMP production in the thyroid gland. PTU inhibits iodide organification and this could explain the elevation in cAMP levels described here. However, PTU could be also acting directly on the phosphodiesterase activity as evidenced by the diminished effects of the drug in the presence of IBMX.

Cells incubated with agents that raised cAMP levels showed an elevation of post-irradiation survival. The effect was greater at higher doses with Forskolin. This difference could be related to the fact that dibutyryl cAMP enters the cell more easily than cAMP (42, 43), but before mimicking cAMP action it suffers one or two deacylation steps (44). On the other hand, Forskolin activates directly adenylate cyclase to produce increasing cAMP levels. Moreover, cell preincubation with H-89, a potent selective inhibitor of cAMP dependent protein kinase A, diminished the radioprotective effects of PTU in FRTL-5 cells. Saavedra et al. showed that cAMP promotes survival through PKA-dependent signaling pathways in Wistar rat thyroid cells (45).

Ionizing radiation is deleterious to living cells because of the structural changes it generates on essential biomolecules. A predominant part of the initial cell damage is caused by the formation of reactive oxygen species including superoxide, hydroxyl radical and hydrogen peroxide, that can interact with almost all cellular components such as DNA, proteins and

lipids leading, if not repaired, to cell death or neoplastic transformation (46). It has been shown that PTU inhibits H_2O_2 generation in neutrophils (47) and in Graves' retro-ocular fibroblasts (48). It also acts as an efficient hydroxyl radicals' scavenger and as an inhibitor of lipid peroxidation in model membranes (49). These antioxidant properties were confirmed by our results showing that pretreatment with PTU resulted in a diminution in ROS levels at an early time after irradiation, although not statistically significantly. Cells have developed effective antioxidant defense systems that include antioxidant enzymes and small molecular weight antioxidants to detoxify free radicals that are continually produced by intrinsic aerobic metabolic processes or after the exposure to ionizing radiation (50). Treatment with PTU resulted in increased activities of antioxidant enzymes after 1 hour in irradiated cells. GPx activity could not be detected in FRTL-5 cells. This fact could be related to the reduced activity of the enzyme observed in this cell line in the absence of a sodium selenite supplement in the culture medium (51). Pretreatment of irradiated skin of mice with the ROS scavenger ascorbic acid resulted in increased SOD and GPx activities after irradiation (52). In agreement with these results, we could hypothesize that pretreatment of cells with PTU could protect them from the damaging action of ROS not only by the direct decrease in ROS levels but also through the elevation of antioxidant enzymes activities.

We evaluated also the protective role of PTU against radiation-induced apoptosis. Cell response after the exposure to ionizing radiation involves mechanisms of cell death as a consequence of oxidative damage to indispensable molecules. PTU pretreatment diminished the number of apoptotic and necrotic cells as seen by morphological nuclei analyses. This could be in agreement with the protection against cellular oxidative damage

explained previously. The diminution in apoptosis is further supported by the fact that pretreatment with PTU partially prevented the loss of the ability to form colonies after irradiation, which is normally due to growth arrest and cell senescence after irradiation (53).

In conclusion, the present study shows that PTU acts as a radioprotector *in vitro* in normal cells possibly, through the elevation of cAMP levels and by a reduction in apoptosis and in radiation-induced oxidative stress damage.

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Author Disclosure Statement

No competing financial interests exist.

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Figure Legends

Figure 1. PTU effects on cellular post-irradiation survival. Cell survival was assessed by the clonogenic survival assay after the exposure to different γ-ray doses**.** Cells incubated with PTU before the irradiation showed an increase in SF2 from 53.3 ± 3.1 (Control) to 74.3 ± 6.3 (PTU) (* $p < 0.05$) (A). MMI and GSH increased SF after irradiation (SF2: Control 50.5 ± 4.1; MMI: 74.6 ± 5.2; GSH 81.8 ± 5.5, $p < 0.05$) (B) while perchlorate had no effect (C) (SF2, Control: 53.4 ± 3.5; $KClO_4$: 58.9 ± 6.1). The resulting points are the average of three independent experiments by triplicate ± SE.

Figure 2. cAMP effect on cell survival. Cells incubated with 1 mM dbcAMP (A) and 10 μM forskolin (B) before the irradiation showed an increase in SF2 from 58.0 ± 2.5 to 67.8 ± 0.4 (** $p < 0.01$) and 55.8 ± 0.8 to 71.1 ± 3.9 (** $p < 0.01$), respectively. Inhibition of cAMP dependent protein kinase A abolished the radioprotective effect of PTU (C). Cell pretreatment with PTU+H-89 before irradiation diminished SF2 to almost control levels abolishing the radioprotective effect of the PTU alone (Control: 53.3 ± 3.9; PTU: 74.3 ± 6.5; PTU+H-89: 57.9 ± 5.3, * $p < 0.05$ *vs* PTU). The resulting points are the average of three independent experiments by triplicate ± SE.

Figure 3. Effect of PTU on radiation-induced oxidative stress. Intracellular ROS levels were measured using the fluorescent probe DCFH-DA 1 and 24 hours after irradiation at the indicated doses. Each value is the average of three experimental determinations by triplicate. Results are expressed as the mean ± SE of each group. * $p < 0.05$ *vs* control.

Figure 4. Effects of PTU on antioxidant enzyme's activities. Cells were irradiated at the indicated doses with or without the addition of PTU. SOD (A) and catalase (B) activities were measured 1 and 24 hours after irradiation, as described in *Materials and Methods*. Each value is the average of three experimental determinations by triplicate. Results are expressed as the mean ± SE of each group. * $p < 0.05$, ** $p < 0.01$, *vs* cells irradiated without PTU; º $p < 0.05$ *vs* control.

Figure 5. PTU effect on radiation-induced cell death. FRTL-5 cells with or without PTU were irradiated with different doses and apoptosis was evaluated after 6, 24, 48 and 72 hours by fluorescence microscopy. Percentage of apoptotic cells (A), or necrotic cells (B) (* $p < 0.05$, ** $p < 0.01$, PTU *vs* irradiated without PTU). C, D, E Representative images from stained cells 24 hours after irradiation. Apoptotic nuclei labelled with Hoechst 33258 (arrowheads) exhibited peripheral chromatin clumping, blebbing and fragmentation (C), cytoplasm of living cells was labelled with DAF (D) and necrotic cells were labelled with IP (E).

Figure 6. Effects of PTU on radiation-induced caspase-3 activity. Caspase-3 activity was measured in the supernatant of FRTL-5 cells at different lengths of time after irradiation, as described in *Materials and Methods*. Each value is the average of three experimental determinations by triplicate. Results are expressed as the mean ± SE of each group. ** $p < 0.01$, *** $p < 0.001$ *vs* non irradiated cells; ºº $p < 0.01$ *vs* cells without PTU.

Footnotes

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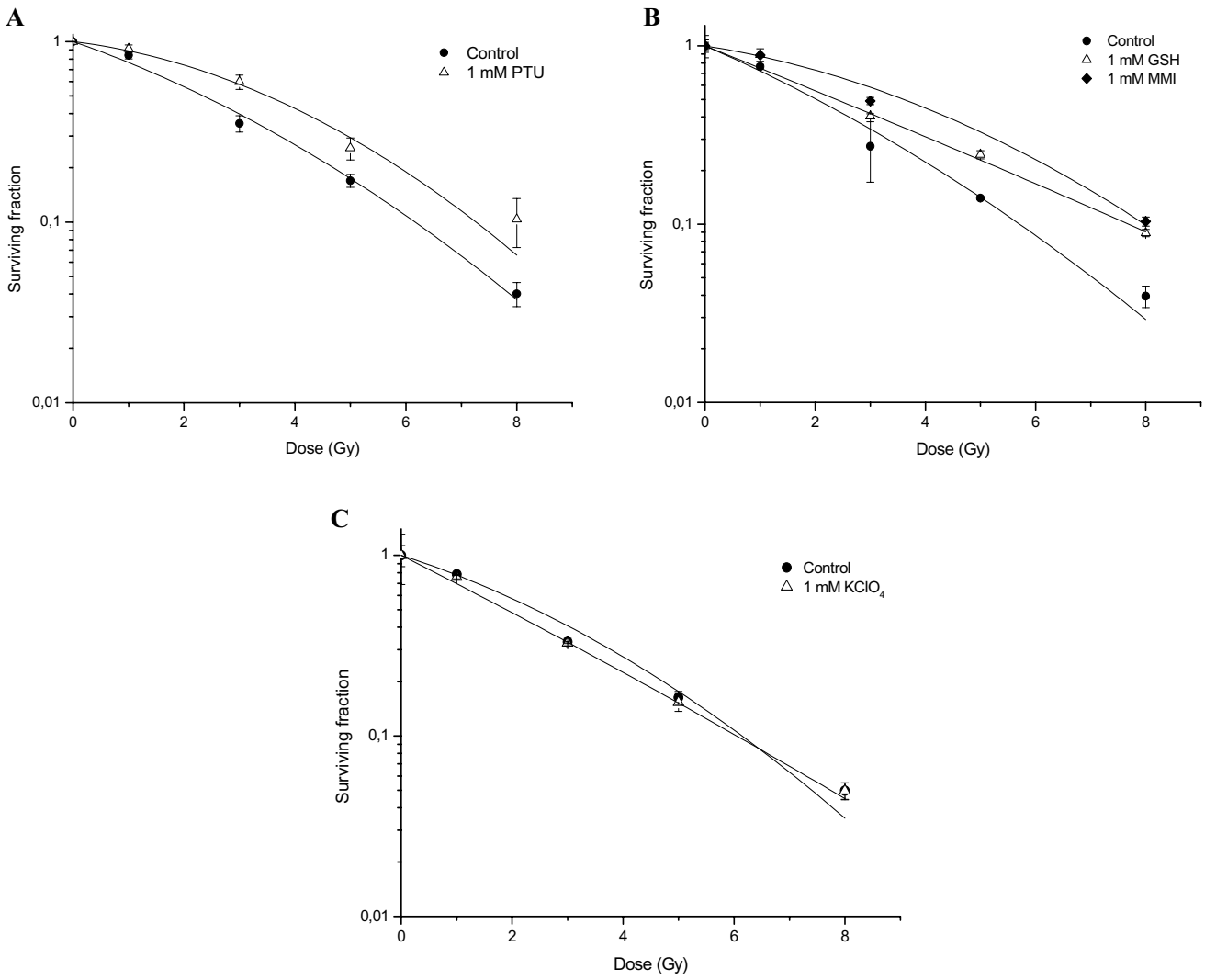
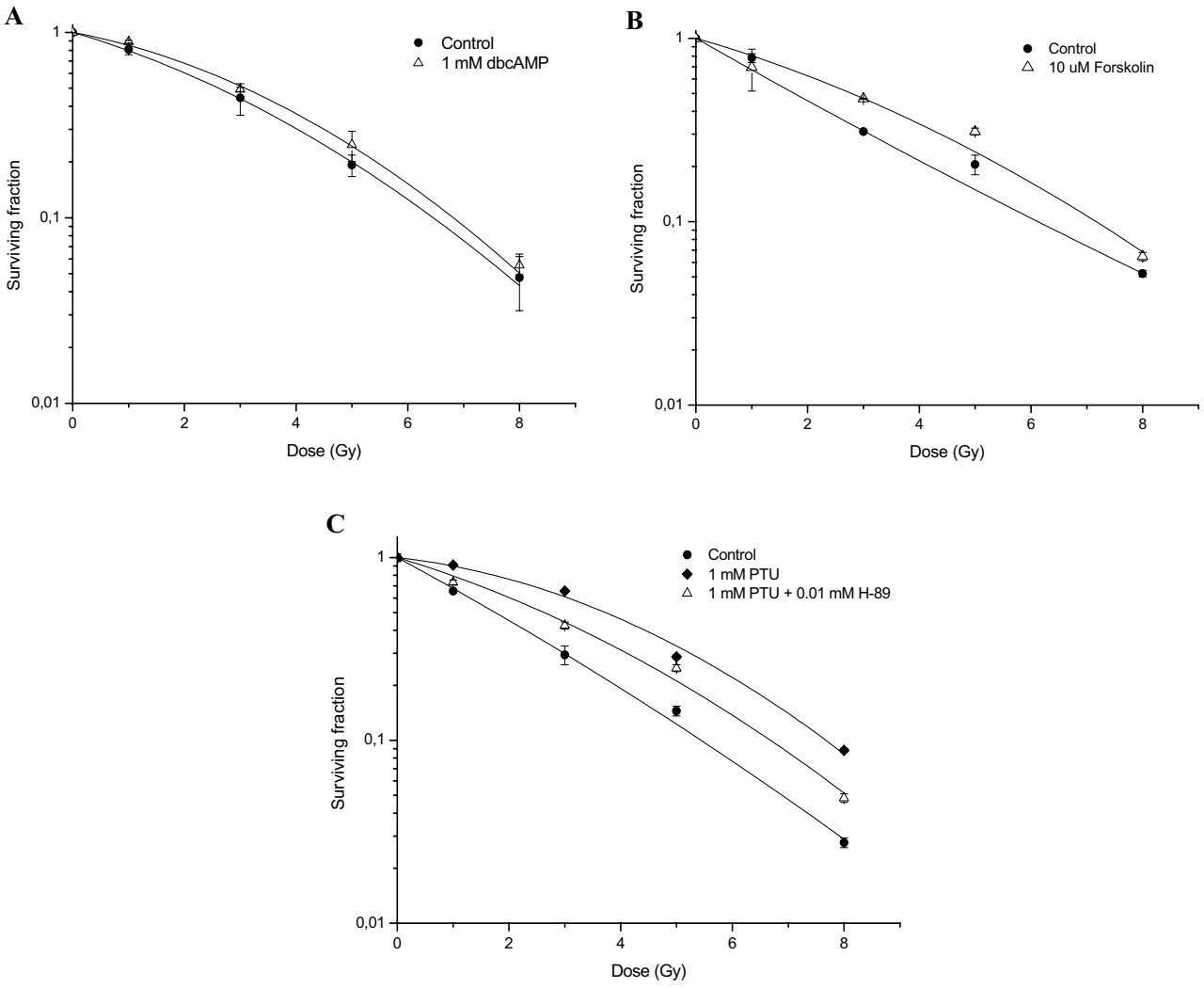
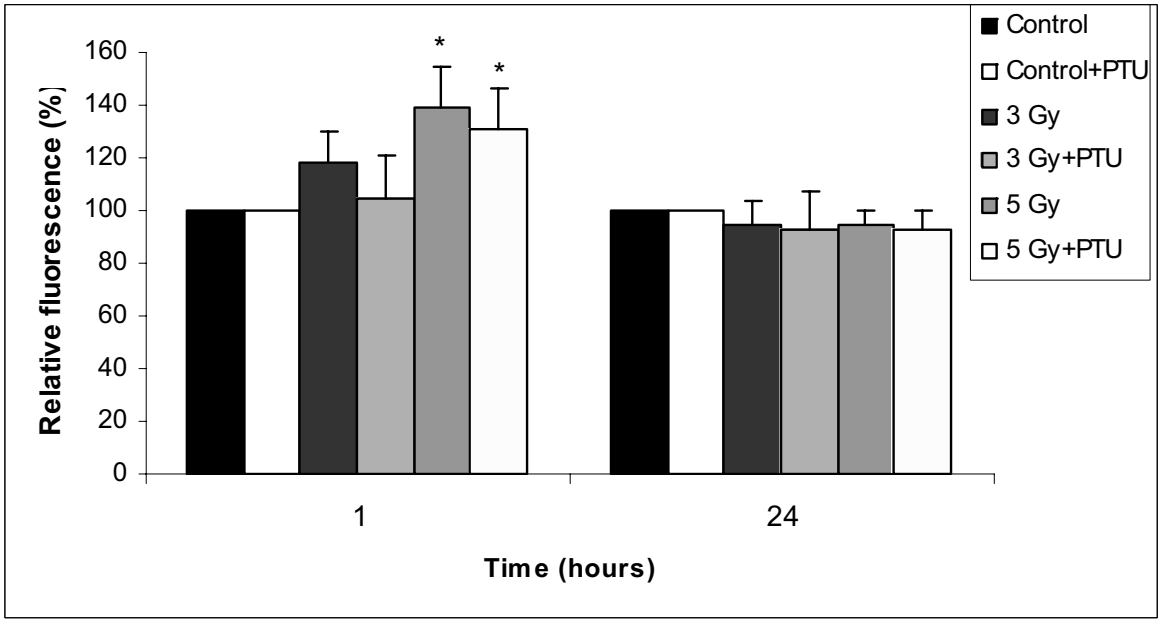
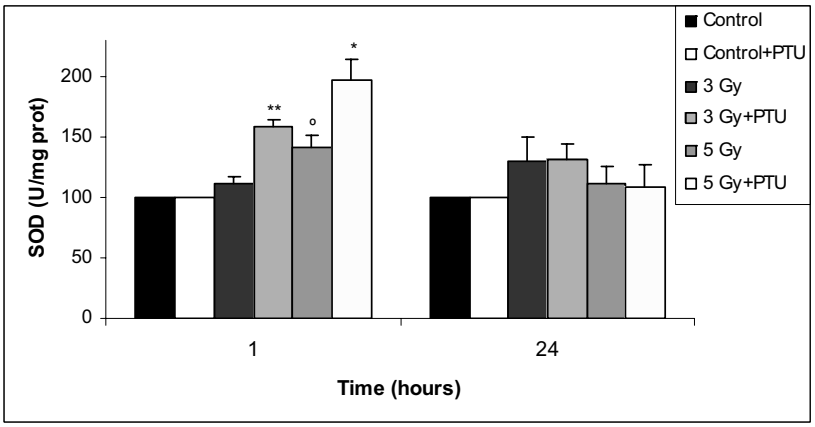

A
Control
1 mM PTU
Surviving fraction
Dose (Gy)
B
Control
1 mM GSH
1 mM MMI
Surviving fraction
Dose (Gy)
C
Control
1 mM KClO4
Surviving fraction
Dose (Gy)

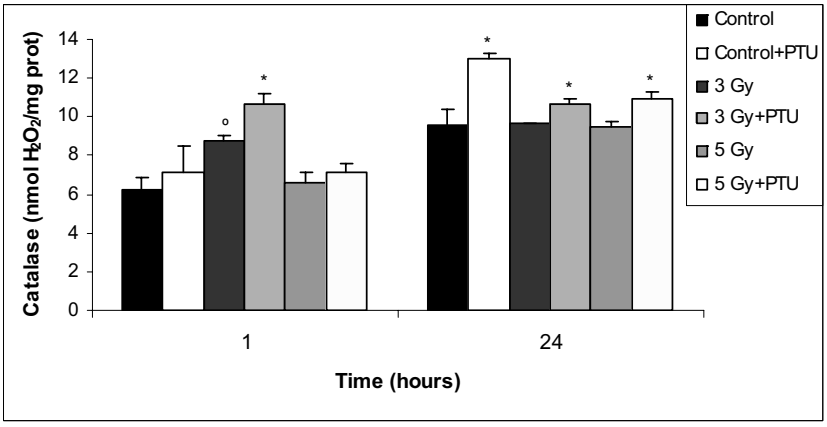

A
Control
1 mM dbcAMP
Surviving fraction
Dose (Gy)
B
Control
10 uM Forskolin
Surviving fraction
Dose (Gy)
C
Control
1 mM PTU
1 mM PTU + 0.01 mM H-89
Surviving fraction
Dose (Gy)


Control
Control+PTU
3 Gy
3 Gy+PTU
5 Gy
5 Gy+PTU
Relative fluorescence (%)
160
140
120
100
80
60
40
20
0
*
*
1
24
Time (hours)

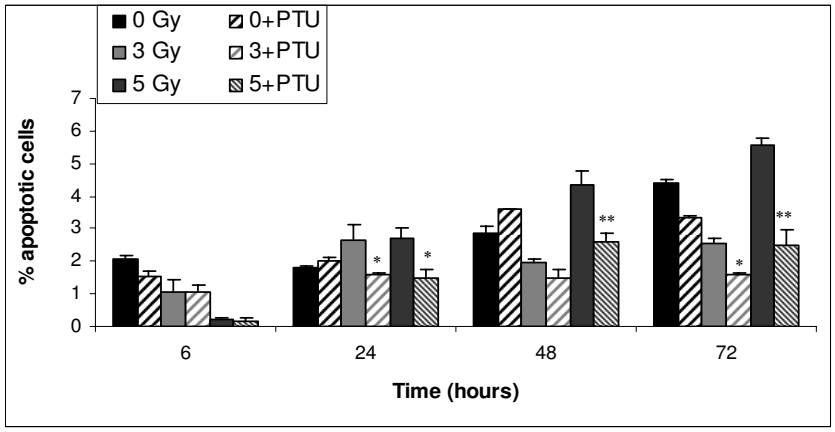
A


Control
Control+PTU
3 Gy
3 Gy+PTU
5 Gy
5 Gy+PTU
SOD (U/mg prot)
0
50
100
150
200
**
°
*
1
24
Time (hours)

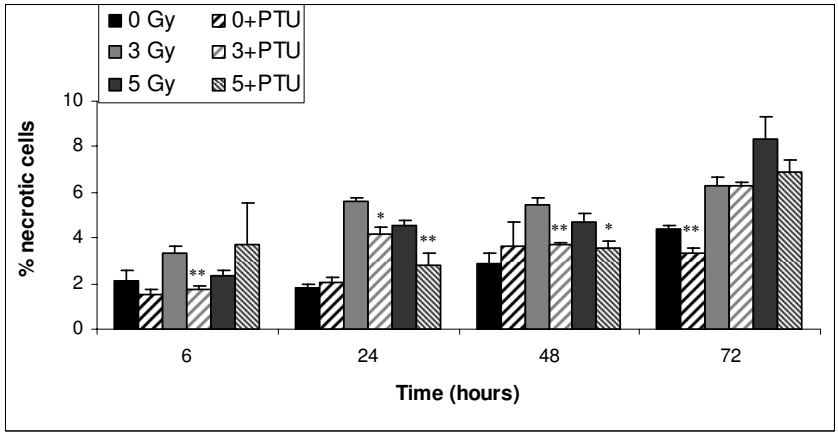
B


Control
Control+PTU
3 Gy
3 Gy+PTU
5 Gy
5 Gy+PTU
Catalase (nmol H2O2/mg prot)
0
2
4
6
8
10
12
14
°
*
*
*
*
1
24
Time (hours)

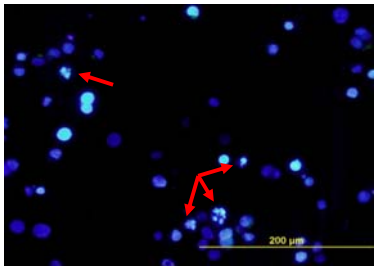
A


0 Gy
3 Gy
5 Gy
0+PTU
3+PTU
5+PTU
% apoptotic cells
0
1
2
3
4
5
6
7
6
24
48
72
Time (hours)
*
**

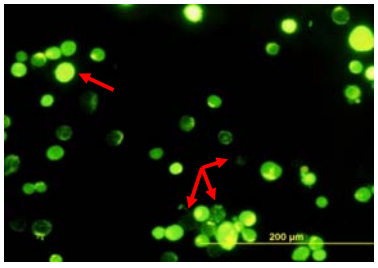
B


0 Gy
3 Gy
5 Gy
0+PTU
3+PTU
5+PTU
% necrotic cells
0
2
4
6
8
10
6
24
48
72
Time (hours)
*
**

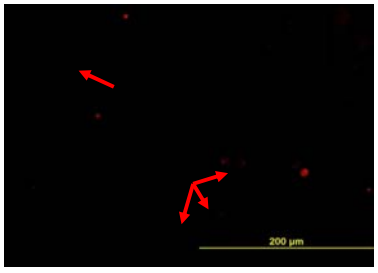
C


200 µm

D


200 µm

E


200 µm

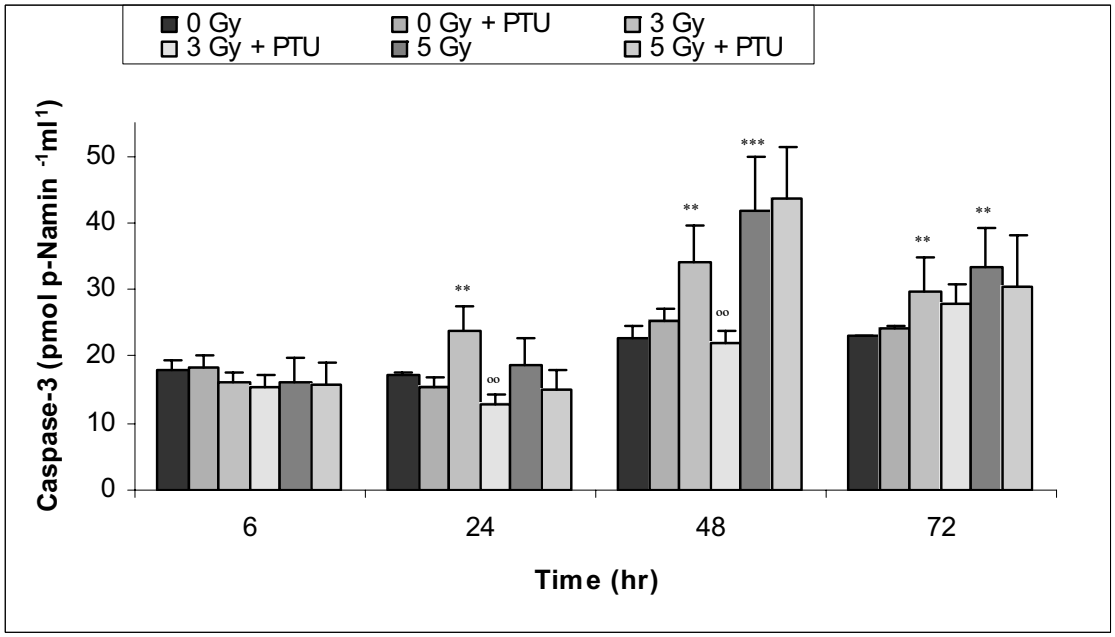

0 Gy
0 Gy + PTU
3 Gy
3 Gy + PTU
5 Gy
5 Gy + PTU
Caspase-3 (pmol p-Namin -1ml1)
0
10
20
30
40
50
6
24
48
72
Time (hr)
**
oo
**
oo
***
**
**

Table 1A. Effect of PTU on cAMP levels.

Time (hr)	Treatment					
	Control	C+IBMX	PTU 0.5 mM	PTU 0.5 mM+IBMX	PTU 1 mM	PTU 1 mM +IBMX
5	1.26 ± 0.08	10.64 ± 0.70	10.54 ± 1.30 **	21.21 ± 1.87 **	23.96 ± 1.80 **	27.12 ± 6.07 **
24	4.95 ± 0.82	41.91 ± 6.01	11.39 ± 2.57 **	49.32 ± 6.00	22.90 ± 2.19 **	48.55 ± 9.41
48	15.63 ± 2.70	117.27 ± 22.40	16.93 ± 1.58	91.69 ± 6.16	23.28 ± 2.32 **	70.53 ± 5.40
72	24.68 ± 2.23	176.63 ± 33.62	38.84 ± 14.89	183.43 ± 9.99	24.23 ± 2.19	83.87 ± 14.28

Table 1B. Effect of different compounds on cAMP levels.

Compound (1 mM)	Treatment	
	Without IBMX	With IBMX
Control	5.1 ± 0.9	52.5 ± 8.2
PTU	16.9 ± 0.2**	58.9 ± 9.3
MMI	5.2 ± 0.7	53.6 ± 2.7
GSH	7.3 ± 0.9	52.6 ± 5.4
$KClO_4$	8.2 ± 1.2	56.5 ± 2.2

(A) Exponentially growing cells were treated with different concentrations of PTU for different hours. (B) Exponentially growing cells were treated with different compounds during 24 hours. Extracellular cyclic AMP levels were measured by RIA. Each value is the average of four experimental determinations by quadruplicate. Results are expressed as the mean ± SE of each group. ** $p < 0.01$ *vs* the respective control.